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Juergen Eckel
Karine Clément *Editors*

From Obesity to Diabetes

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Preface: From Obesity to Diabetes

Obesity is a major risk factor for the development of type 2 diabetes and its associated complications, representing a major socio-economic burden for healthcare systems. The worldwide prevalence of obesity doubled since 1980, and as a consequence, the number of patients with diabetes has been continuously rising with more than 450 million people suffering from this disease at the present time. Substantial progress has been made in understanding the molecular pathways leading from excessive fat accumulation to metabolic perturbations and finally diabetes manifestation as well as related comorbidities. Nevertheless, innovative treatment regimens are urgently required to cope with the epidemic increase of obesity-associated complications.

This edition of the *Handbook of Experimental Pharmacology* aims to analyze new insights into the pathophysiology of obesity, to examine some organ alterations and perturbed inter-organ cross-talk, to decipher the complex links to diabetes and its complications, and to collect most recent information on new strategies for the prevention and treatment of obesity and diabetes. Leading experts in the field of obesity and diabetes research provide deep insights into the current state of the art and have made substantial attempts to emphasize future directions. Individual chapters address the critical role of obesity and specifically the intestine and adipose tissue for the pathophysiology of diabetes and its complications, cover different approaches of lifestyle modifications, and highlight most recent developments of novel drugs for obesity and diabetes. Indeed, for years, only a few pharmacological solutions were available for patients with obesity, but this is changing rapidly with new molecules in the academic and pharmaceutical pipelines that will be available in the near future and hopefully change patients' lives.

We are confident that the current collection of papers will contribute to a deeper understanding of the critical role of obesity for a variety of metabolic diseases, and may at the same time stimulate future discussion and decision making regarding the development of innovative drugs and therapeutic approaches.

Düsseldorf, Germany — Juergen Eckel
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Epidemiology of Obesity

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Abstract

Obesity is in theory defined on the basis of the excess health risk caused by adiposity exceeding the size normally found in the population, but for practical reasons, the World Health Organization (WHO) has defined obesity as a body mass index (weight (kg)/height $(m)^2$) of 30 or above for adults. WHO considers the steep increases in prevalence of obesity in all age groups, especially since the 1970s as a global obesity epidemic. Today, approximately 650 million adult people and approximately 340 million children and adolescence (5–19 years) suffer from obesity. It is generally more prevalent among women and older age groups than among men and younger age groups. Beyond the necessity of availability of food, evidence about causes of obesity is still very limited. However, studies have shown that obesity 'runs in families', where both genetics and environmental, and especially social, factors play important roles. Obesity is associated with an increased risk of many adverse medical, mental and social consequences, including a strong relation to type 2 diabetes. Type 2 diabetes and related metabolic syndrome and diseases are major contributors to the excess morbidity and mortality associated with obesity.

Keywords

Causality · Epidemic · Metabolic syndrome · Morbidity · Mortality · Obesity · Type 2 diabetes

1 Introduction: Why Obesity Matters

The ability to store fat as an energy reserve ready for future use is ubiquitous in the nature and essential for survival and reproduction of many species. However, excess fat stores, defined as obesity, in humans are associated with an increased risk of multiple adverse effects on physical and mental health and on social life. Hippocrates observed the health risk associated with obesity about 2,400 years ago, and during the last century, numerous large-scale studies have confirmed the association by showing that the greater the degree of obesity, the higher is the excess mortality (Aune et al. 2016; Global B. M. I. Mortality Collaboration et al. 2016). Recently, using modern technics for causal inference such as instrumental variable analysis, including Mendelian randomisation, the association of BMI with mortality has proven to be causal. Thus, it is appropriate to refer to the association as effects of obesity on mortality (Sun et al. 2019).

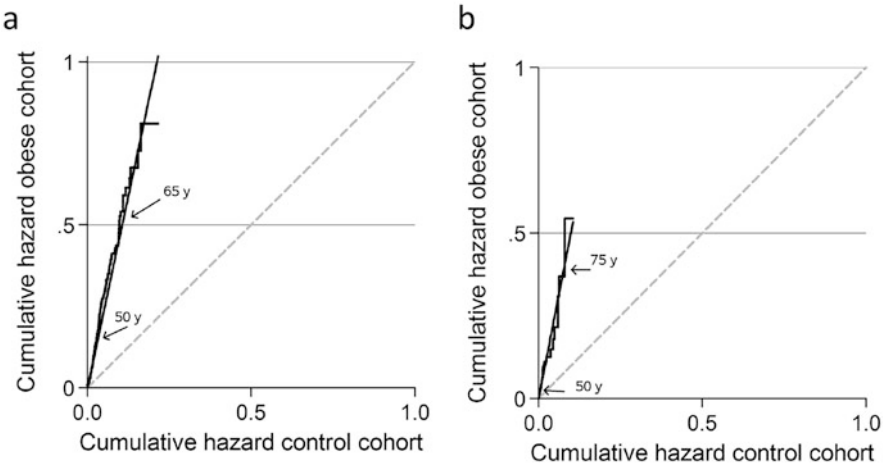

Fig. 1 Cumulative hazard of diabetes for men entering adult life with obesity vs. a randomly sampled control cohort from the same Danish population, and followed up at corresponding ages from 18 through 80 years. (**a**) First incidence of diabetes. (**b**) Prevalent diabetes at time of death. Note the constantly elevated hazard ratio of 4.9 (95% confidence intervals 4.1–5.9) for the first incidence and 6.8 (4.6–10.1) for diabetes at the time of death. Figure adapted with permission from Zimmermann et al. (2011)

Concerning the current topic of the book, it is particularly relevant to emphasise the relation of obesity to type 2 diabetes. Thus, obesity is associated with increased risk of a series of metabolic aberrations, constituting the 'metabolic syndrome', predisposing to (or in some definitions of the syndrome including) type 2 diabetes and its associated co-morbidities, especially the micro- and macro-vascular diseases (Després et al. 2008). The risk of diabetes throughout life relates strongly to presence of obesity at entrance to adult life (Fig. 1) (Zimmermann et al. 2011). These metabolic conditions are major contributors to the excess all-cause mortality that unequivocally follows the presence of obesity (Bhaskaran et al. 2018).

This chapter will primarily address various challenging aspects of obesity epidemiology and is far from being a comprehensive account of the topic. We refer readers with interest in more detailed and referenced account of the established background knowledge to the available handbooks and other publicly available sources (Hu 2008; UK Government's Foresight Programme 2007; Bray and Bouchard 2014; WHO 2021).

2 Definition of Obesity by Its Relation to Health Effects

While considering 'adiposity' as the trait of body fat mass, the definition of obesity is in theory based on the excess risk of adverse health effects following a certain degree of adiposity exceeding the size normally found in the population. However, in

practice, such dichotomous definition is inoperable for several reasons. First, it would require precise measurements of the degree of adiposity in large populations followed over many years to estimate the risks of diseases associated with different degrees of adiposity. Although technically feasible, too few and too small studies are available to allow adequate quantitative estimation of the risk. Second, adiposity is a continuous trait that appears to have a smooth, monotonically rising association with the adverse health effects. Third, other features of adiposity than just total amount of fat have health impacts, such as the size of the depots at different sites, including ectopic sites, adipocytes size and numbers (hypertrophic vs. hyperplastic adipose tissue), changes over time in size of fat mass, and duration of the current state of adiposity. In addition to this heterogeneity, there are other obesity-related factors that influence the effects of size and type of adiposity on various health outcomes, such as various lifestyle components, e.g. physical activity, dietary and drinking habits, smoking, etc. The multiple sources of this heterogeneity, both at the population and individual level, make it arbitrary and dubious to set a fixed threshold for defining obesity.

In spite of the problems mentioned above, public health practice, codified by W. H.O., has adopted a fixed threshold of obesity based on body mass index (BMI = weight (kg)/(height (m))^2), assuming BMI to be a reasonably valid and reliable measure of degree of adiposity. This may be true on an average group basis (WHO 2021). In multiple large-scale cohort studies, published during the last five decades, the relation between BMI in adulthood and all-cause mortality exhibits a U- or J-shaped association with the bottom most often in the BMI range of 20–25 (Aune et al. 2016; Global B. M. I. Mortality Collaboration et al. 2016). From BMI of around 30 and above, there is a distinct monotonous upward trend in all studies, which has made W.H.O. choose this level for the official definition of obesity or 'extreme overweight' (with moderate overweight being defined as BMI in the interval 25 through 30) among adults (WHO 2021). Due to the changing body proportions during growth in childhood, this BMI-based definition cannot be used in children, in whom sex- and age-specific values must be used.

In global public health settings, however, there are concerns about the applicability of these thresholds. The thresholds originate from studies of the populations in the Western countries, leading to too high thresholds for the Asian populations in particular, where the increased morbidity and mortality emerge at lower BMI. Moreover, the thresholds may be too low in older populations in general. People with obesity who have been able to survive to older ages do not have the similarly increased future risk as those who entered adult life with obesity. Finally, it is clear that when dealing with individual patients or peoples, BMI is far too crude as a measure of adiposity. In other words, some people with high BMI may have little and insignificant adiposity, whereas some people with low BMI may suffer from relatively high degree of adiposity, causing significant health problems.

Although BMI-based criteria for obesity cannot be used in children and adolescents, evidence shows that excessive adiposity and corresponding BMI also during these ages have adverse long-term health effects, including increased risk of diabetes (Zimmermann et al. 2017; Hudda et al. 2021). However, the long-term

effects only appear if the condition persists to the entrance to adult life (Bjerregaard et al. 2018, 2020). Moreover, weight gain from normal levels after entrance to adulthood may be associated with the same greater risks later in life as if the obesity status had persisted since entrance to adult life (Black et al. 2005). Thus, the main reason for an association of obesity in childhood and adolescence with the later adverse health effects appears to be the correlation with obesity in adulthood (Aarestrup et al. 2016). The exception is that some children and adolescents with massive obesity do develop the metabolic aberrations and metabolic-syndrome-associated diseases, especially type 2 diabetes, already during this age period.

3 Obesity Subtypes, Body Fat Distribution, Ectopic Depositions

The distribution of the fat mass on various sites in the body shows a considerable individual variation, and the distribution changes with aging. The primary site of fat deposition is in the subcutaneous area, but even in this body compartment, the distribution is different between individuals. The two genders show clear differences, where women tend to accumulate fat in the gluteo-femoral regions, hence called the *gynoid* type, and men tend to accumulate fat in the truncal, and mainly in the abdominal area, hence called the *android* type. This latter pattern often accompanies an excess accumulation of fat within the abdominal cavity, in the mesenterium, the omentum, and the retroperitoneal area. Ectopic fat deposition, i.e. deposition in organs not usually storing excess amount of fat, may occur within the liver (fatty liver disease), but also in other organs, e.g. the skeletal muscles cells and the pancreas, including the islets.

Simple measurements may probe the patterns of body fat distribution; waist circumference is a proxy measure of the android type, and hip or thigh circumference is a proxy measure of the gynoid type. The waist-to-hip ratio expresses the relative predominance of these body types. Normally, men would have a ratio around 1.00, and women around 0.85. The proxy nature of these measures implies that a high waist circumference may be due to both intra- and extra-abdominal fat accumulation, with likely different health effects. Moreover, large hip circumference may be due to both the shape of the skeleton and muscles and the size of the fat mass deposition in this region.

These gender differences are only tendencies; women may develop the android type, especially after the menopause, and men may also develop the gynoid type. In many people with obesity, there is just an overall excess of fat accumulation with little or no gender-specific pattern. However, the body fat distribution matters with regard to metabolic adverse health effects (Després et al. 2008; Christakoudi et al. 2020). The android type is associated with exaggeration of the adverse effects. This is likely driven primarily by the metabolic states manifest in ectopic fat deposition, whereas little if any adverse metabolic effects are associated with the gynoid type. These differences in effects show up in the excess mortality associated with obesity. Nonetheless, irrespective of the type of obesity, the overall association between

increased fat mass and adverse health effects, including excess mortality, remains (Christakoudi et al. 2020).

4 Prevalence and its Dependencies of Multilevel Geography, Sex and Age

According to a recent global estimate ~650 million adult people (out of 5.5 billion people) have obesity, defined by BMI of 30 and above (NCD Risk Factor Collaboration (NCD-RisC) 2017). Using the internationally accepted criteria for obesity in childhood and adolescence, estimates of the number of individuals aged 5–19 with obesity is ~340 million. As shown in the global map (Fig. 2), the distribution of people with obesity exhibits great variation between countries. Generally, obesity is often more prevalent among women than men and often more prevalent in older than in younger age groups. In women, the prevalence of obesity ranges from 2.7% to 65.3% (Table 1) and in men from 1.7% to 59.9% (Table 2). There were 36 countries where the prevalence of obesity among women was above 35%, including the USA, South Africa, a number of countries in Polynesia and Micronesia, the Middle East and northern Africa. In contrast, there were only 14 countries where the prevalence of obesity among men was above 35%, including the USA and a number of countries in Polynesia and Micronesia, the Middle East and northern Africa. However, in the

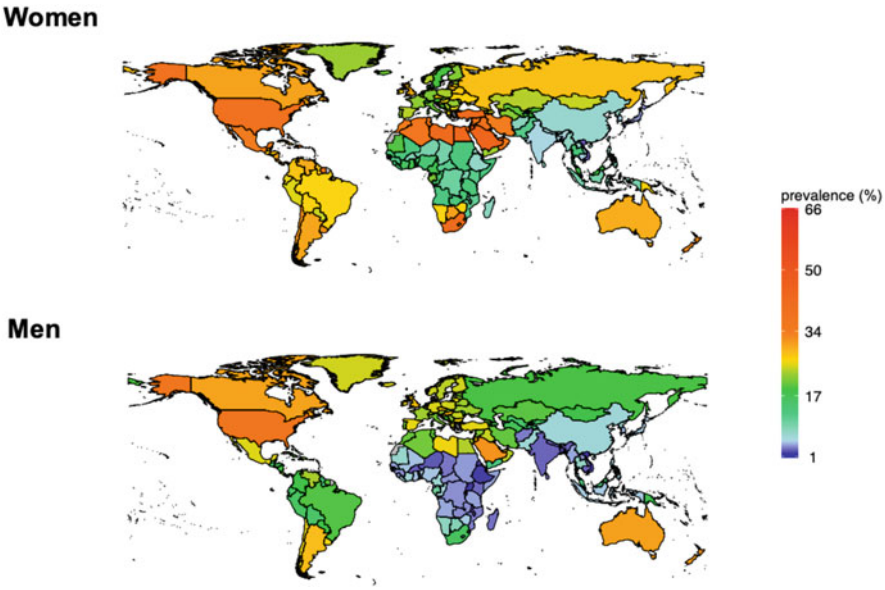

Fig. 2 Prevalence of obesity in women and men by country across the world in 2016. Data are based on estimates provided by the NCD-RisC collaboration (https://ncdrisc.org/) (NCD Risk Factor Collaboration (NCD-RisC) 2017). Note the great heterogeneity between continents, between countries within continents and even between the small ocean islands listed below the maps

Table 1 Mean BMI and prevalence of obesity in women in 2016 in countries, five in the bottom, five in the middle, and five in the top ranks among the 200 countries across the world

Country	Mean BMI (kg/m^2)	Obesity prevalence (%)	Rank (1–200)
Viet Nam	21.94	2.71	1
Japan	21.82	3.86	2
South Korea	23.16	5.03	3
Cambodia	22.39	5.06	4
Timor-Leste	21.37	5.15	5
...	...	...	...
Norway	26.26	23.48	98
Kazakhstan	26.67	23.65	99
Spain	24.93	23.78	100
Armenia	27.19	24.02	101
Montenegro	26.30	24.10	102
...	...	...	...
Marshall Islands	30.67	59.02	196
Palau	29.72	60.48	197
Cook Islands	33.36	60.85	198
Nauru	32.95	64.81	199
American Samoa	35.15	65.32	200

Data are based on estimates provided by the NCD-RisC collaboration (https://ncdrisc.org/)

Table 2 Mean BMI and prevalence of obesity in men in 2016 in countries, five in the bottom, five in the middle, and five in the top ranks among the 200 countries across the world

Country	Mean BMI (kg/m^2)	Obesity prevalence (%)	Rank (1–200)
Viet Nam	21.97	1.67	1
Uganda	21.61	1.92	2
Ethiopia	20.11	1.98	3
Rwanda	21.28	2.01	4
Eritrea	20.32	2.13	5
...	...	...	...
Paraguay	26.71	17.80	98
Bosnia and Herzegovina	26.53	17.82	99
Mongolia	26.05	18.24	100
Colombia	25.99	18.28	101
Panama	26.33	18.48	102
...	...	...	...
Marshall Islands	29.08	49.85	196
Palau	29.64	53.15	197
Cook Islands	32.86	53.97	198
American Samoa	33.07	58.75	199
Nauru	32.28	59.85	200

Data are based on estimates provided by the NCD-RisC collaboration (https://ncdrisc.org/)

two biggest countries in Asia and the Western World, the gender differences were limited: in China, the obesity prevalence was 6.8% among women and 6.1% among men, and in the USA the prevalence was 38.2% among women and 36.5% among men. The heterogeneity in occurrence of obesity is also considerable between various regions within countries (Centers for Disease Control and Prevention 2021), even in regions of countries considered culturally and socially fairly homogenous, like those in Scandinavia.

In an attempt to understand the occurrence of obesity and explain the heterogeneity of its occurrence, the following sections address aspects of causes of obesity and its development.

5 The Energy-Balance Theorem: Eaten Too Much and Moved Too Little

The first thermodynamic law about energy conservation implies that changes of amount of energy in a closed space can only happen by corresponding changes in input of energy minus output of energy from the closed space. The prevailing understanding of the development of obesity, manifest in body weight gain, is to consider the body as such closed space to which there has been an input of energy in excess of output. While the input of energy to the body is food, the output of energy is expenditure of energy that ends up in either dissipation of heat produced by the metabolism (whether basic or by body movements) or excretions of energy in the urine and faeces. If outside forces push more food into the body than the total output of energy, the content of energy in the body will increase correspondingly.

Whereas the energy-balance theorem is obviously a correct description of the quantities of changes of energy in the body during body weight changes (not due to changes in water contents), it is essential to the application of the theorem to consider the consequences of the body being a living entity rather than a dead space. The theorem does not inform about which causal mechanisms make the changes, i.e. whether the primary driver is excess intake relative to output, or whether the primary driver is an internal increase in stored energy that elicits an excess intake relative to output of energy (Sørensen 2009, 2014). One very common line of thoughts holds that excessive intake of calories beyond the current needs of energy in the metabolism drives the deposition of the surplus of energy as fat in the adipose tissue, and, if continued, leads to the development of obesity.

Although this contention is in accordance with the findings that voluntary experimental overfeeding may elicit some, individually varying, enlargement of the fat mass, the findings are fundamentally different from the obesity phenotype. In the typical progressive obesity phenotype, a weight loss induced by reduced food intake will typically later result in regain of body weight, whereas the reduced food intake after cessation of experimental overfeeding leads to shrinking fat mass and no tendency to regaining body weight. This difference suggests that some forces of fat deposition other than increased food intake drive the obesity phenotype. In principle, it may well be that altered internal body processes result in a primary

accumulation of energy as fat in the body. The accumulation of fat may subsequently induce increased input of energy to the body by eating more, and/or decrease output of energy by moving less or slowing down the metabolism to secure the energy supplies to the body metabolism overall.

It remains unknown in which causal directions the mechanisms are operating, and it is heavily debated what these mechanisms could be (Speakman and Hall 2021; Ludwig et al. 2021). Thus, it is a major task for the future obesity research to identify which energy fluxes drives the development of obesity. This knowledge will have profound implications for how to control the fluxes. If obesity originates from primary excess input of calories, then the task is to reduce the energy input. On the other hand, if the primary process is the enhanced storage of fat, then reducing energy input would not reduce the fat depots, instead it would lead the body to continuously strive to refill the reduced depots.

The complexity and challenges to explain the causes of obesity became apparent when a large group of UK researcher attempted to map the system of factors supposed to determine obesity development some years ago. The map entails several different domains with multiple internal relationships between many components, all presumed implicated in directly or indirectly driving the individual energy balance to the positive side (Fig. 3) (UK Government's Foresight Programme 2007). As mentioned, it remains an open question if this model with a core engine regulating energy balance by passive accumulation of a surplus of energy as fat in the adipose tissue is right.

A key challenge in elucidation of the causal mechanisms is that the amount of energy deposited in the adipose tissue per day during obesity development is very small (usually $<1\%$ of total intake). This makes the process difficult to monitor, and the only way to determine it at the moment is to observe the enlargement of the adipose compartment over prolonged periods. Another challenge is that in parallel with the growing fat mass, also the metabolically active lean body mass becomes bigger, and its growth and concurrent energy-demand is far greater than the accumulating energy as fat. Moreover, the energy spend on physical activity does not decline (although the level of physical activity may decline, according to Newton's law due to the greater energy requirement to move the greater body mass). Overall, this implies that people who are developing obesity must eat much more to serve the derived needs of energy in the lean body mass than people who are not developing obesity (Sørensen 2009). Availability of enough food to support this process is therefore a permissive condition facilitating the development of obesity. On the other hand, it is not an absolute prerequisite for obesity development. It may occur in spite of limited access to food, demonstrated in animal experiments and clearly seen in some of the rare monogenic forms of obesity in both animals and humans. Yet, it is likely also possible in humans in more general forms, in which case the forces of fat deposition outweigh the needs of energy for the rest of the body.

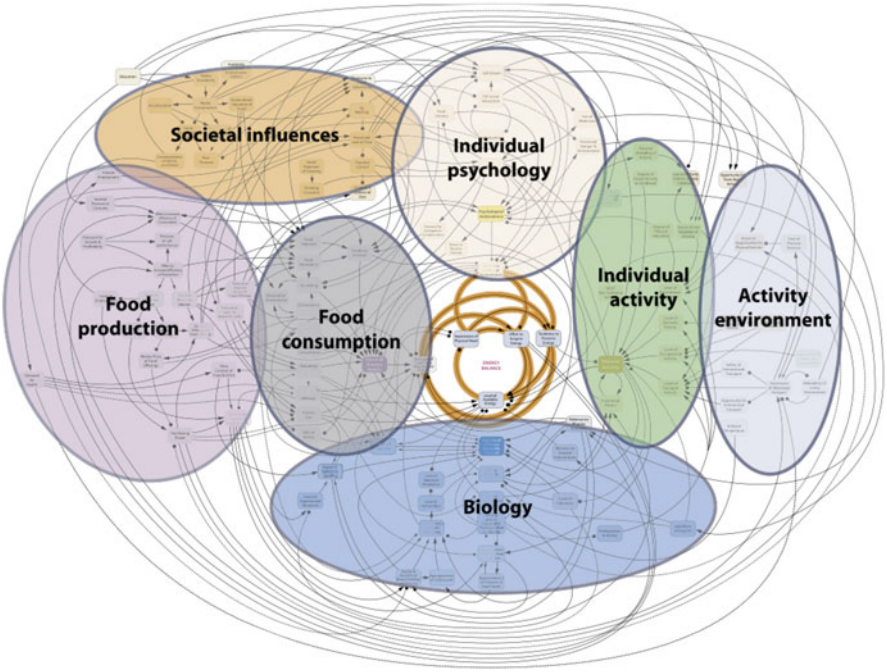

Fig. 3 An obesity system map outlining the putative factors pertaining to several different domains (labelled in the lower right corner), directly or indirectly influencing eventually the energy balance in the centre. This map highlights the factors and interconnections of particular relevance in children. Figure adapted with permission from UK Government's Foresight Programme (2007). Readers interested in the specific factors included in the map may consult the original report

6 Within and Between Population Risk Factors for Obesity

Many factors influence changes in BMI and the size of fat mass over time. These factors are often equated with risk factors of obesity, making the implicit assumption that if these factors continue their actions over longer time, often many years, it will eventually result in obesity, but such long-term associations are rarely, if ever, observed. Changes in individual BMI over time may originate from changes in eating and exercise, which are similar in origin to those seen in the voluntary overfeeding studies. However, since the overfeeding studies do not produce the obesity phenotype as explained above, the changes in BMI following changes in eating and exercise habits may not apply to risk of obesity. To claim that a factor that influences body weight or fat mass sizes also is a risk factor for obesity will require studies, which shows such associations over the long time it takes to develop obesity.

While aiming for the simplicity of the most parsimonious understanding of the causes of development of obesity, we will need to acknowledge the possibilities that difference in occurrence of obesity may originate from differences in sets of factors

that together constitutes sufficient sets for obesity development. This applies to differences between individuals within populations, differences between different time points in the same population, and differences between populations.

If we imagine the apparently broad panel of factors that may lead to development of obesity in a single individual (Fig. 3), it is clear that we may question which factors are necessary, which are sufficient, which are influenced by or even conditional on other factors as mediators or modifiers of causal pathways (UK Government's Foresight Programme 2007). Currently, our knowledge about the factors unfortunately is far too limited to make such classification of the pathways beyond the trivial claim about necessity of some food intake, but even that factor needs qualification to accommodate the abovementioned findings of the possible growth of the fat mass during food restrictions.

7 Familial Risk, Genetics and Shared Environment

It is very old knowledge that obesity 'runs in families', which means that for persons with obesity, the likelihood that other persons in the family also have obesity is greater than for persons without obesity. Generally, BMI exhibits a familial correlation indicating that the broad range of the continuous trait explains why obesity 'runs in families'. The magnitudes of these effects vary by type of family relationship (parent-offspring, siblings, twins etc.), possible environmental modifications of the genetic expression (gene by environment interactions), age at assessment and definitions of obesity, but surprisingly not by gender.

During the last several decades, multiple studies have aimed at disentangling the role of the genes transmitted within the biologically related family members from the role of the environment shared by the family members, whether biologically related or not, in creating the familial occurrences in BMI and obesity. Under reasonable assumptions about the structure of genetic transmission (additive, non-additive, dominant or recessive) and shared environments, the role of genes and environment may be estimated from nuclear families (parents and offspring). Yet, the main contribution to solve the problem has been application of adoption and twin studies, which may complement each other by resting on different assumptions. The degree to which individuals adopted-away very early in life resemble their biological family members in BMI may express the genetic influence, and, correspondingly, the resemblance with the adoptive family members may reflect the influence of the shared environment. In twin studies, the difference in resemblance of BMI between monozygotic and dizygotic twin pairs is due to half of the genetic effects, whereas what remains of resemblance between the pairs not attributable to genetic effects is due to their shared environment. What remains of phenotypic variation not attributable to the genetic and shared environmental influence provides an estimate of the influence of the non-shared (unique or specific) environment to the individual differences.

It is important to notice that these divisions of contributions to the phenotypic variance apply to members of the same population. Thus, differences between

different populations may well be due to differences in the general exposure of the entire population to environmental influences as well as possible genetic differences. Rapid changes in the phenotypic distribution in one population over time is most likely due to rapid changes in the environmental exposures, which is typically what has happened in the obesity epidemic.

The key message emerging from the few adoption and many twin studies (Silventoinen et al. 2010, 2016, 2017; Elks et al. 2012) is that the resemblance in BMI of biological family members no longer sharing the household environment is entirely attributable to genetic influences. The shared environment does have some, though weaker, influence while individuals still live in the same household, typically kids until they reach early adulthood (Silventoinen et al. 2016). This is in contrast to the prevailing expectation that habits influencing BMI adopted in the shared environment are to be responsible for the effects carried through to the life outside the household environment. The changes in level of genetic influence on BMI across the ages during childhood and adolescence may indicate that the age-driven biological processes of the body influences the expression of the genes at specific ages. However, the influence of shared environment also changes during childhood and adolescence. Thus, the greater age difference between siblings, the lower are the correlations of BMI between them (Rokholm et al. 2013).

8 General – Family Independent – Environmental Risk Factors

The distribution of the family-independent differences in exposures to environmental factors takes place in the populations through other channels than what creates differences between family members, although the exposures may well affect all family members. However, a key challenge is that it is very difficult to explore what these causal environmental factors are, because of the very slow developmental process of obesity and the possible changes in the exposures alongside the development of obesity.

When occurrence of obesity changes over time, both in individuals and in populations, where it is assumed that no changes in genetics happen, the changes must be explained by changes in the environmental exposures. A general and reasonable assumption is that no major genetic changes can occur at the population level during the short period of the obesity epidemic, although some changes in principle could occur due to changes in assortative mating and because of migrations between populations with different genetic loads for obesity. On the other hand, environmental exposures may interact with the genetics implying that the effects on obesity possibly depend on the individual genetic profile. While the overall variation in BMI increases during the development of the obesity epidemic, so do both the proportion of variance attributable to genetics and to environmental influences (Fig. 4). However, the ratio between these components (the heritability) as well as the familial correlations remains stable (Ajslev et al. 2014, 2015). The increasing genetic variance means that the changes in the environment over time operate in such

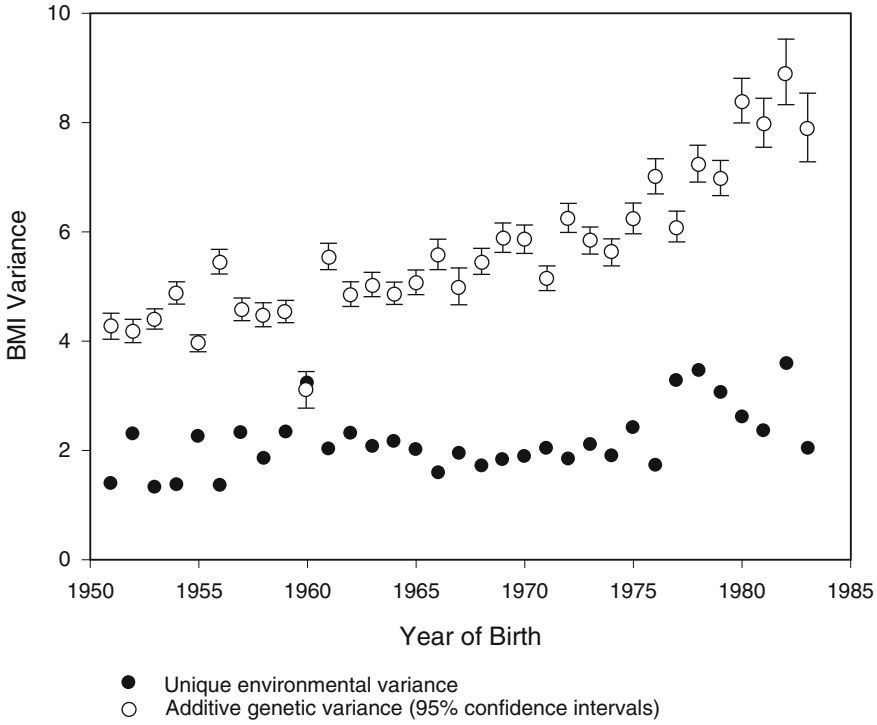

Fig. 4 Proportions of BMI variation explained by additive genetic and specific (unique) environmental factors using data from ~1.5 M Swedish conscripts at age 18–19 years born between 1951 and 1983. Note the high level of genetic influence across all cohorts. Figure reproduced with permission from Rokholm et al. (2011)

a way that it allows a stronger expression of the genetic predisposition (Silventoinen et al. 2017; Rokholm et al. 2011).

9 Risk Factors vs. Modifiable and Non-modifiable Causal Factors of Obesity

A risk factor for obesity is a variable that is statistically associated in a presumed unbiased way with presence of or development of obesity in the population under study. However, such associations may not reflect causal associations between the risk factor and obesity. The associations may instead reflect confounding, where another variable is the causal factor, which also is related to the risk factor of interest, and hence creating a non-causal association with the risk of obesity development. Reverse causation may bias the observed risk factor because development of obesity along the way may alter the risk factors suspected to promote it, e.g. obesity leads to increased food intake and reduced physical activity. Identified causal factors may or

may not be modifiable for principal or practical reasons, which determines the utility of the knowledge about the causal factor. As explained above, there is no doubt that differences in genetic and environmental factors play a causal role in development of obesity, but the modifiability is unknown.

There is a long list of empirically shown or suspected risk factors for obesity (Fig. 3), but rather few have proven to be causal factors (UK Government's Foresight Programme 2007). Whereas it apparently may be helpful to subdivide the specific risk factors in genetic and environmental factors, this is a far too simple dichotomy. Most of the presumed environmental factors are behavioural factors, e.g. eating and drinking habits, smoking, and physical activity, which by themselves have both genetic and environmental roots in the individuals, their ancestors, and the general environment around them. The same applies to the panel of psychosocial and socioeconomic factors, e.g. both own and parental education may be a result of combined influences of genetics and environments. The possibility that particular genetic background may lead the persons to seek or make particular environment, e.g. drawing people genetically predisposed to obesity to seek environments where there is abundance of cheap, tasty, and energy-dense food available further enhances the complexity.

While genetic factors, broadly defined, play a causal role, the question is which of the specific DNA elements are responsible for the effects on obesity. Besides the very rare forms of monogenic obesity, all the many ~1,000 common genetic variants (single nucleotide polymorphisms (SNPs)) of the genome that have been statistically associated with BMI, obesity or obesity types are not proven to express causal relationships (Loos and Yeo 2022). The SNPs may be located in the neighbourhood of the causal variants, which will make them statistically associated with obesity, because of the lower likelihood of splitting the DNA string between them during formation of the gamete cells (meiosis). The challenges in attributing specific genetic differences to a causal role are further leveraged by the fact that only a small fraction of the phenotypic variance can be explained by the so far identified SNPs. However, progress is emerging in understanding the role of the specific genetic variants (Loos and Yeo 2022). Attempts to localise where in the body the neighbouring causal genes are expressed points for most of the genes associated with BMI to various sites in the brains, whereas the adipose tissue expresses the genes associated with obesity types (Locke et al. 2015). The recent progress in understanding how the first discovered genetic locus associated with common obesity operates illustrated the complexity of the task. It is still the strongest and includes a cluster of SNPs in the first intron of the *FTO* gene, which appears to operate in combination with another set of genes at some distance (*IRX3* and *IRX5*), through different pathways (Sobreira et al. 2021).

10 Trans-Generational and Perinatal – Epigenetically Mediated – Risk Factors

The individual risk of development of obesity may depend on exposures early in life, possibly even before conception by exposures to the parents and grandparents. This effect rests on the possibility that environmental exposures to the parents and grandparents may leave the so-called epigenetic marks on the DNA of the gametes transmitted by conception to the next generation. The susceptibility to epigenetic modification of the DNA mainly happens during the formation of the gamete cells, which for men is a continuous process in the testicles during adult life, but for women this is during formation of the eggs in foetal life. Therefore, it is the exposures to the grandmothers during her pregnancy with the forthcoming mother that may be relevant. So far, there is emerging, but still little evidence supporting such effects.

The epigenetic modifications may as well take place during foetal life or postnatal life. Parental smoking before and during pregnancy is strongly and likely causally associated with later risk of obesity in the children (Morgen et al. 2018; Albers et al. 2018; Philips et al. 2020), and maternal smoking after childbirth may also influence the risk (Møller et al. 2014). The many epigenetic marks caused by smoking, also during pregnancy (Joubert et al. 2016), may mediate the effects the children.

Birth weight is positively associated with later BMI and occurrence of obesity, but the overall association is composed of associations in opposite directions, likely reflecting that birth weight is a combined and complex phenotype resulting from numerous genetic and prenatal environmental influences (Morgen et al. 2017; Warrington et al. 2019; Rugholm et al. 2005). Low birth weight (for given gestational age) is associated with an increased tendency to develop abdominal obesity and metabolic complications, including diabetes, later in life (Huang et al. 2019). Smoking during pregnancy reduces birth weight, followed, as mentioned, by increased risk of obesity later during childhood. If the forthcoming mother develops gestational diabetes, the child may have high birth weight and may show an increased risk of obesity, but the presence of obesity in these mothers likely explains the association (Patro Golab et al. 2018).

Children who have been breastfeed experience less obesity later in life than children who have not been breastfeed, and the association appears stronger with increased duration of breastfeeding, but the evidence is ambiguous (Cope and Allison 2008). Timing and content of the complementary feeding may influence later risk of obesity. Paradoxically, infant feeding of a diet low in fat and high in protein may promote later development of obesity (Rolland-Cachera et al. 2016).

For all these early life maternally influenced risk factors, it is important to control for genetic confounding, i.e. the genetic transmission from mother to child of predisposition to obesity. Thus, maternal variables influenced by obesity, e.g. psychosocial and socioeconomic status, smoking habits, pregnancy complications modulating birth weight up or down, breastfeeding initiation and duration, and choice of composition of the complementary and later feeding may create associations with childhood obesity that are due to or inflated by genetic

confounding. To assign a lasting causal effect of these early influences, it is essential to show that the associations are not created by the transmission of genetic predisposition to obesity (Parsons et al. 1999).

11 Life-Course Approach, Life-Long Challenges

In a group of people followed for a long period, the BMI at one age is correlated with BMI at later age, which is called tracking correlation and is based on the tendency of individuals maintaining their mutual position in the BMI distributions. When individuals change their mutual position in the BMI distribution in the population, it weakens the tracking correlations, wherefore the tracking correlations decline by increasing time intervals between measurements. BMI and obesity show tracking throughout the life course, i.e. that BMI at any given age, starting at birth, correlates with BMI at later ages (Aarestrup et al. 2016; Rugholm et al. 2005). The basis for tracking BMI is the unaltered genetic influences and/or persistence in effects of environmental exposures (Silventoinen and Kaprio 2009). The environmental exposure could act continuously or could have acted in a limited time window earlier, perhaps in prenatal or even pre-conceptual life, as discussed, with persistent effects on the phenotype. Individual departures from the tracking must indicate changes in some environmental exposures. Generally, there is little tendency to regress to previous tracking levels, which implies that the prevalence of obesity will increase by age as it does in most populations.

Development of obesity during childhood shows a pattern denoted 'early adiposity rebound', which suggests that the first years after infancy is a critical age window (Rolland-Cachera et al. 2016). In the normal development of infants, they accumulate excessive fat depots during the first year, which thereafter disappears the following year. At later preschool ages the children start again to accumulate fat – the 'adiposity rebound' – more so in girls than in boys, and in girls it continues through puberty, whereas it stops before puberty in boys. However, in children who later develop obesity, this accumulation starts earlier, possibly stimulated by the feeding of excessive protein and little fat in the first years of life (Rolland-Cachera et al. 2016).

12 Multilevel Social Risk Factors

Since Goldblatt, Moore and Stunkard in 1965 published their seminal study on social factors in obesity (Goldblatt et al. 1965), numerous studies have confirmed the inverse association between the individual social position and the risk of obesity (Fig. 5) (Wang and Beydoun 2007; Shrewsbury and Wardle 2008; El-Sayed et al. 2012a, b; Hoebel et al. 2019). This is typically present in high- and middle-income countries. In low-income countries, the higher prevalence of obesity may be found in the upper social classes, likely attributable to food deprivation in the lower classes in these societies. The inverse association is usually steeper for women than for men.

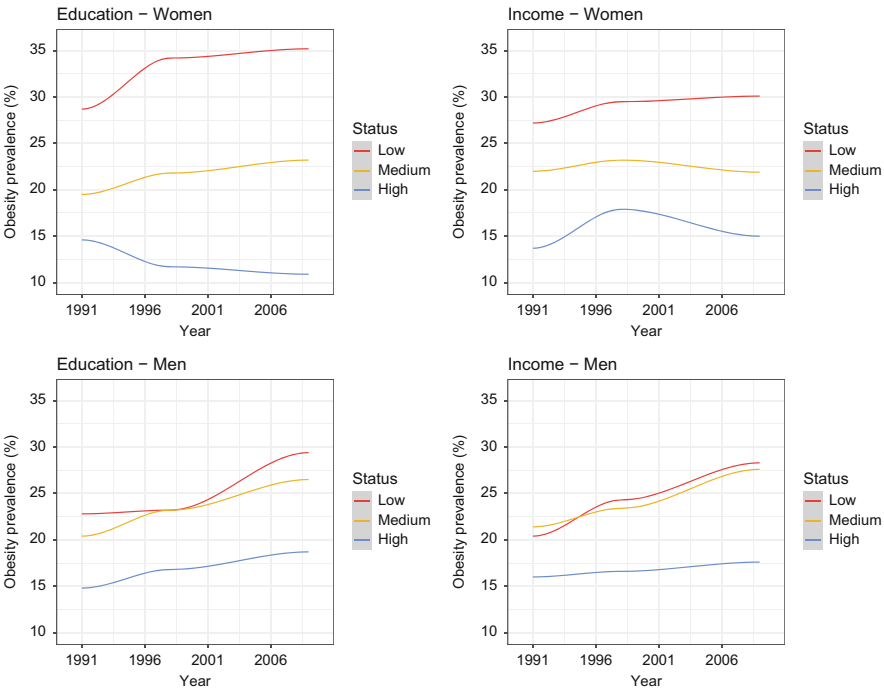

Fig. 5 Trends on age-standardised prevalence of obesity among adults aged 25–69 years over time for different socioeconomic groups, using national examination data from Germany. Note that in each survey period, obesity was least prevalent in the highest socioeconomic groups for both genders. The figure represents results shown in Table 2 in Hoebel et al. (2019)

The parental social position is inversely associated with obesity in children. The inverse relation of obesity with the social position emerges by several different ways of probing it, e.g. by duration of education, by hierarchical position of the occupation, and by economic wealth including income, fortunes and housing. The inverse association is manifest at various group levels, such as between neighbourhoods, parities, municipalities, and regions of different socioeconomic status. Overall, the social factors appear to be the strongest and most dominant risk factor for obesity at the population levels. The question is whether some form of chronic stress exposure and/or obesogenic behaviours mediates the relationship and whether intervening to reduce the socioeconomic inequality would reduce obesity (Moore and Cunningham 2012; Hillier-Brown et al. 2014). Stress exposures before conception, during gestation, and during childhood may have lasting effects on later risk of development of obesity.

Given this pattern of results, the question is whether the effect of the genetic predisposition depends on the social environment. Generally, it appears as if the lower social position allows greater genetic influence or, in other words, that high social position tends to suppress the genetic influence (Silventoinen et al. 2019). However, the social position may influence the risk of obesity, independent of the

genetic and shared environmental influences, manifests in the familial correlations of BMI. An adoption study showed that the social position of the adoptive parents maintains an influence on the BMI of the adult adoptees even though there is no relation to the BMI of the adoptive parents (Teasdale et al. 1990). On the other hand, parental social factors may also be an integrated part of the genetic transmission, as revealed by analyses of the social position and educational level of the biological parents (Fontaine et al. 2011).

13 Development of the Obesity Epidemic and Future Predictions

The fact that the prevalence of obesity in children, adolescents and adults has increased quite rapidly over time in many countries since the Second World War and nearly tripled since 1975 has led to the notion of the obesity epidemic (Fig. 6) (NCD Risk Factor Collaboration (NCD-RisC) 2017). Total body fat, estimated from skinfold thicknesses, showed the same trend (Olds 2009). However, there has been a great heterogeneity in the development of the obesity epidemic across the

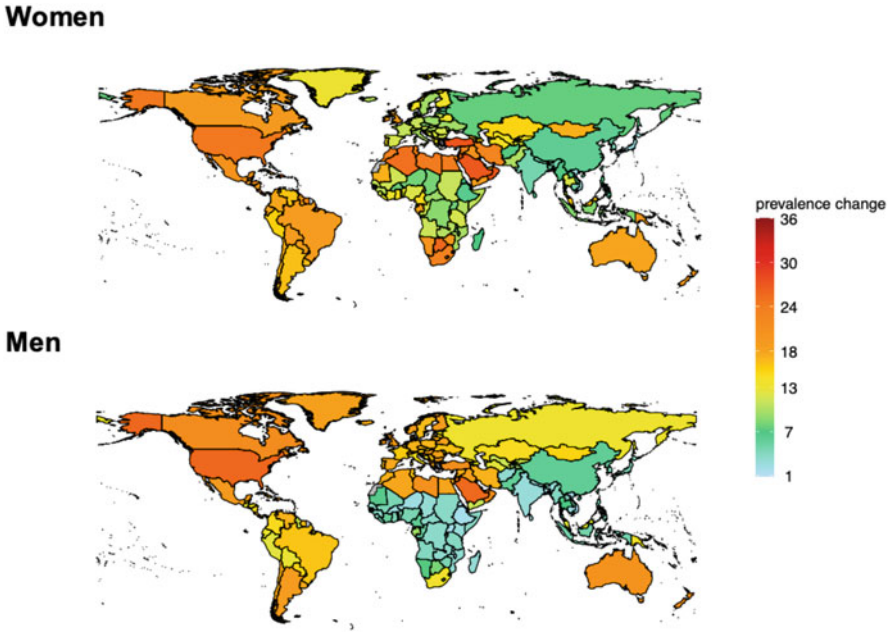

Fig. 6 Changes in prevalence of obesity in women and men from 1975 to 2016 between countries across the world. Note that also the changes in prevalence like the prevalence in 2016 (see Fig. 2) show great heterogeneity; between continents, between countries within continents and even between the small ocean islands listed below the maps. Data are estimates provided by the NCD-RisC collaboration (https://ncdrisc.org/) (NCD Risk Factor Collaboration (NCD-RisC) 2017)

Table 3 Changes in obesity prevalence from 1975 to 2016 in women in countries, five in the bottom, five in the middle and five in the top ranks among the 200 countries across the world

Country	Prevalence (%) 1975	Prevalence (%) 2016	Prevalence change (%)	Rank (1–200)
Singapore	4.71	6.63	1.92	1
Japan	1.49	3.86	2.37	2
Viet Nam	0.22	2.71	2.49	3
South Korea	0.84	5.03	4.19	4
Cambodia	0.42	5.06	4.64	5
...	...	...	...	...
Gambia	1.73	15.46	13.73	98
Albania	8.99	22.72	13.73	99
Netherlands	6.97	20.86	13.88	100
Norway	9.48	23.48	14.00	101
Uzbekistan	5.84	19.86	14.02	102
...	...	...	...	...
Tonga	27.59	56.06	28.47	196
Kiribati	21.10	51.96	30.86	197
Niue	25.49	56.77	31.28	198
Tokelau	19.51	52.18	32.67	199
Tuvalu	24.57	57.85	33.28	200

Data are based on estimates provided by the NCD-RisC collaboration (https://ncdrisc.org/)

200 countries who have reported changes in the prevalence of obesity between 1975 through 2016. The differences in prevalence percentages between these years range from 1.9% to 33.3% in women (Table 3) and from 1.6% to 35.5% in men (Table 4). In China, the prevalence increased by 6.0% in women and 5.9% in men, whereas the prevalence in the USA increased by 24.3% in women and 25.8% in men.

The heterogeneity in the changes over time is mainly due to differences in the positions of the BMI distributions (NCD Risk Factor Collaboration (NCD-RisC) 2021), and the rise in prevalence of obesity is mainly occurring in the rural areas (NCD Risk Factor Collaboration (NCD-RisC) 2019). However, a disproportionately strong rise has taken place in the extreme upper tail of the distribution, both in cities and rural areas, but much more pronounced in the rural areas (Sonne-Holm and Sørensen 1977). The heterogeneity applies as well to changes in BMI and prevalence of obesity in childhood and adolescence, as well as to the changes among adults in the same populations (NCD Risk Factor Collaboration (NCD-RisC) 2017).

Since the turn of the century, there has been a levelling off in the rise in many of the Western countries among children and adolescents, whereas the increase continues among adults and in most other parts of the World (NCD Risk Factor Collaboration (NCD-RisC) 2017; Rokholm et al. 2010). Although less investigated, there is evidence from some areas that the rise in obesity prevalence began slowly around the Second World War (Olds and Harten 2001; Olsen et al. 2006). The rise in prevalence among children and young adults seems to have occurred in two phases, linked to birth cohorts, i.e. the same increasing phases occurred among individuals

Table 4 Changes in obesity prevalence from 1975 to 2016 in men in countries, five in the bottom, five in the middle and five in the top ranks among the 200 countries across the world

Country	Prevalence (%) 1975	Prevalence (%) 2016	Prevalence change (%)	Rank (1–200)
Viet Nam	0.06	1.67	1.60	1
Uganda	0.17	1.92	1.75	2
Ethiopia	0.18	1.98	1.80	3
Rwanda	0.12	2.01	1.89	4
Eritrea	0.20	2.13	1.93	5
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Papua New Guinea	3.37	17.26	13.88	98
Honduras	2.14	16.24	14.10	99
Italy	6.67	20.94	14.27	100
Kazakhstan	4.88	19.57	14.69	101
Nicaragua	3.79	18.61	14.82	102
...	...	...	...	...
Kiribati	12.43	42.87	30.44	196
Palau	22.58	53.15	30.56	197
Cook Islands	22.49	53.97	31.48	198
Niue	14.18	46.17	31.98	199
Tuvalu	12.96	48.47	35.50	200

Data are based on estimates provided by the NCD-RisC collaboration (https://ncdrisc.org/)

born in the same years irrespective of the years of measurement. The first phase occurred in the first post-war decade, and following a phase of a stable period of about two decades; the second, and steeper, phase occurred from the 1970s and onwards (Fig. 7) (Rokholm et al. 2010). The causes of these irregular developments are unknown, but their identification may provide an opportunity to reverse them.

Predictions of the future trends in prevalence of obesity must imply some form of extrapolations from the current levels combined with future expectations based on the preceding trends. A crucial question has been whether linear extrapolations would be valid, and if not, which deviations from linearity would be justified. The irregular development of alternating phases of stability and increases in the countries where there are longest known history of the obesity epidemic obviously makes any predictions very uncertain. Yet, it seems likely that the rising trends in most countries will continue for a while (NCD Risk Factor Collaboration (NCD-RisC) 2017).

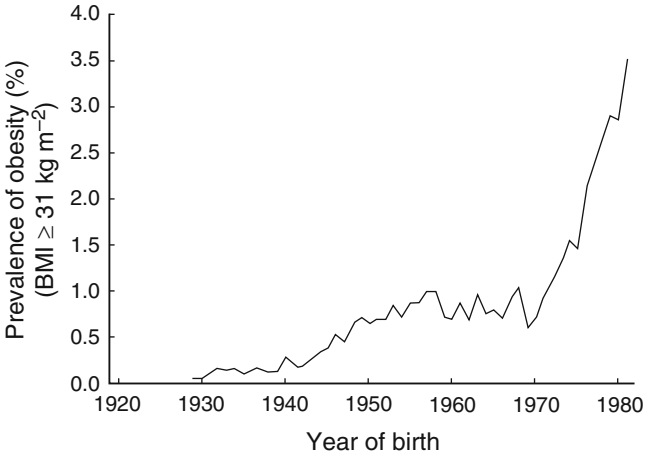

Fig. 7 Trends in prevalence of obesity (here defined as BMI $\geq$ 31.0 kg/m^2) in Danish young men measured at the mandatory draft board examination by year of birth from 1930 to 1980. Note that the prevalence increased in two phases, the first one through the 1940s, and the second one through the 1970s, with a plateau in prevalence before both phases. Figure reproduced with permission from Rokholm et al. (2010)

14 Presumed Drivers of the Obesity Epidemic

Whereas the individual development of obesity must require multiple components in the causal mechanisms and possibly different sets of components in different individuals, changes in some particular components may have driven the obesity epidemic. It is possible that just a few or even a single component of the entire panel of causal components have enhanced the risk of individual development of obesity (Sørensen et al. 2012). Secular changes in exposures of the populations to many other putative causal factors have been on the research agenda for decades, but the evidence for their roles in driving the epidemic remains elusive (McAllister et al. 2009).

The obesity epidemic is usually attributed to the increasingly 'obesogenic environment' that many populations have experienced in the last several decades, but beyond referring to the 'environment' as opposed to the presumed unchanged genetic predisposition, this claim is uninformative unless the specific components of the environment are identified. The prevailing contention is that these components are the increasingly easy and cheap access to energy-dense, highly processed, tasty, so-called junk food, and sugar-sweetened beverages, likely combined with multiple mechanical devices that reduces the needs to be physically active both at work and in leisure time and in transportation (McAllister et al. 2009). A steadily increasing number of people in the populations have changed their lifestyles to an apparently obesogenic behaviour by accepting these foods, drinks and devices. However, the interpretation of the role of the obesogenic environment and behaviours must

consider the above arguments about the possible underlying causal mechanisms of the disturbed energy balance. Considering the obesogenic environment as defined here, it is clearly an important permissive condition, resulting in increased occurrence of obesity, but only if other factors, such as the genetic and social factors, are also in action.

15 Epilogue

There are several open questions about the epidemiology of obesity and the underlying biology. How are the causal mechanisms that produce an enlarged fat mass and what determines whether it has implications for the risk of metabolic co-morbidities and when this emerges? A comprehensive overview of risk factors vs. modifiable and non-modifiable causes of obesity would be very valuable. Which are the causal genetic and environmental factors that make up the familial concordances in obesity? Which causes operate independent of the families? What is driving the inverse association of social factors with obesity, and why are they so dominating? Can the distribution of social factors explain the great heterogeneity in occurrence of obesity between populations? What is the time relationship between causes and effects? Are there early life or even transgenerational exposures to causal factors that leave effects to be manifest many years later? Which causes have changed and thereby induced the obesity epidemic, and what are the predictions about their future actions? Can modifications of these causes help to mitigate the obesity epidemic or its consequences of possibly invalidating and fatal co-morbidities?

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The Circadian Clock and Obesity

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Contents

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Abstract

The modern way of life has dramatically affected our biological rhythms. Circadian rhythms, which are generated by an endogenous circadian clock, are observed in a large number of physiological functions including metabolism. Proper peripheral clock synchronization by different signals including appropriate feeding/fasting cycles is essential to coordinate and temporally gate metabolic processes. In this chapter, we emphasize the importance of nutrient sensing by peripheral clocks and highlight the major role of peripheral and central clock communication to locally regulate metabolic processes and ensure optimal energy storage and expenditure. As a consequence, changes in eating behavior and/or bedtime, as occurs upon shift work and jet lag, have direct consequences on metabolism and participate in the increasing prevalence of obesity and associated metabolic diseases such as type 2 diabetes and non-alcoholic fatty liver disease. In this setting, time-restricted feeding has been suggested as an efficient approach to ameliorate metabolic parameters and control body weight.

Keywords

Chrononutrition · Circadian clock · Metabolic diseases · Obesity

1 Introduction

The last decades have witnessed a sharp rise in metabolic diseases prevalence. In 2015, obesity was affecting 39% of the population worldwide due to a sedentary lifestyle combined with increased consumption of industrial foods enriched in refined carbohydrates and fat (Chooi et al. 2019). Obesity is accompanied by a greater risk of developing other pathologies such as type 2 diabetes (T2D), cardiovascular and non-alcoholic fatty liver (NAFLD) diseases. Thus, obesity has become a major public health challenge requiring appropriate medical intervention.

Our environment is governed by cyclic rhythms linked to the layout of our solar system. For instance, ambient temperature and the photoperiod fluctuate according to the seasons. Circadian rhythms are biochemical, physiological, and behavioral cycles that oscillate with a period of about 24 h to align with the light/dark cycle due to the rotation of the earth around its axis. These endogenous rhythms are generated by the biological clock which is an ancestral system present in all organisms, from cyanobacteria to humans, allowing them to anticipate predictable environmental changes such as the day/night cycle. In mammals, the body has to carry out a large number of biological functions over the course of the day such as regulation of wakefulness and sleep, secretion of hormones, control of body temperature, metabolism, cell division, and DNA repair. The temporal organization of all these functions is essential to coordinate them and allow them to be activated within the right time window.

In humans, the modern lifestyle has considerably altered biological rhythms. Physical activity, feeding, and exposure to light are no longer limited to daylight hours (i.e., natural light), sleep duration has been considerably reduced, and around 20% of the working population are shift workers. In addition, changes in sleep patterns between the worked days and days off, also defined as social jet lag, affect 2/3 of the population. In this chapter, we focus on the interaction between the clock and metabolic processes and describe several contexts in which clock disruption contributes to the development of metabolic pathologies such as obesity. Finally, we discuss how dietary interventions such as time-restricted feeding/eating (TRF/TRE) may help manage body weight and improve metabolic health.

2 The Biological Clock

Circadian rhythms are generated by a network of biological circadian clocks, which, in mammals, include a so-called central clock located in the suprachiasmatic nuclei of the hypothalamus, each consisting of about 10,000 neurons (Moore and Eichler 1972; Stephan and Zucker 1972) and peripheral clocks located in all tissues of the body such as the heart, the liver, the muscle, the lung, the skin, etc. (Mohawk et al. 2012). A fundamental feature of this circadian system is its endogenous and autonomous rhythmic activity. Consequently, in a constant environment devoid of external time cues, circadian rhythms arise with a period of about 24 h (on average 24.2 h in humans) (Duffy et al. 2011). Therefore, the clock needs to be entrained daily by external factors to adjust to 24 h. In mammals, the primary environmental time cue is the light detected by melanopsin-containing retinal ganglion cells. Light information is then converted into electric pulses transferred to the suprachiasmatic nuclei through the retino-hypothalamic axis. The suprachiasmatic nuclei then synchronize peripheral clocks through the sympathetic and parasympathetic nervous system or the secretion of hormones such as glucocorticoids. Food intake and exercise are also potent synchronizing signals for some peripheral clocks which enables effective energy storage and expenditure. This will be further detailed below.

3 The Molecular Clock Machinery

3.1 Core Clock Transcriptional Regulation

At the molecular level, the biological clock consists of transcription factors exerting positive and negative feedback on each other, thus generating self-sustaining molecular oscillations that control the cellular electrical and biochemical activities with a period close to 24 h (Reppert and Weaver 2002). The transcription factors Clock and Bmal1 form the positive arm of the molecular clockwork. They bind as heterodimers to consensus sequences in the regulatory regions of the *Per, Cry, ROR, and Rev-erbs* target genes to activate their transcription (Gekakis et al. 1998). Per and Cry, once in sufficient quantity, heterodimerize through their PAS domain and enter the nucleus

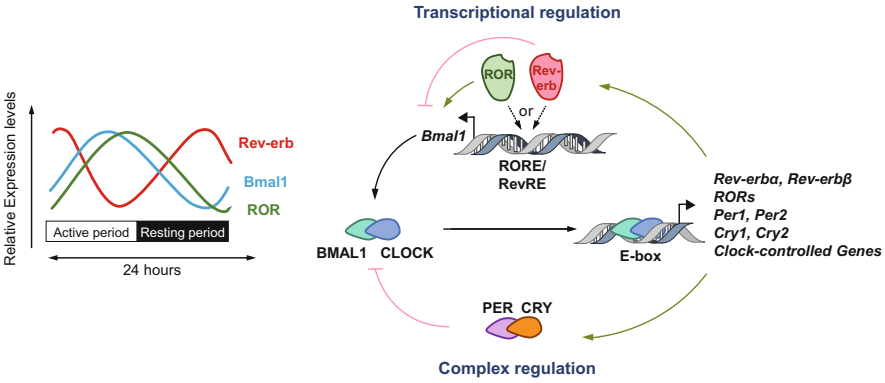

Fig. 1 The clock machinery. The molecular clock is composed by transcription–translation feedback loops. The transcription complex BMAL1/CLOCK induces the expression of E-box-containing genes including the negative regulators Period (PER) and Cryptochrome (CRY (right panel)). In turn, the PER/CRY heterodimer inhibits the transcriptional activity of BMAL1/CLOCK. Once PER and CRY levels are sufficiently low, a new cycle may start. CLOCK/BMAL1 induces the expression of the nuclear receptors Rev-erbα/β and retinoid-related orphan receptor α, β, and γ (RORα/β/γ). Rev-erbs and RORs interact with co-repressors (NCoR) and co-activators (NCoA) and compete for the binding of RevRE/RORE elements in common target genes to repress or activate, respectively, their transcription. The overall effect of these loops is the rhythmic expression of these factors (as shown on the left panel), thus generating a circadian expression pattern

to inhibit the transcriptional activity of *Clock* and *Bmal1*, hence their own transcription. The nuclear receptors RORs and Reverbs compete to activate and inhibit Bmal1, respectively (Guillaumond et al. 2005; Preitner et al. 2002). These transcriptional feedback loops result in circadian oscillations in clock gene expression (Fig. 1).

3.2 Post-Translational Clock Regulation

In addition, clock proteins are subject to numerous post-translational modifications that alter their stability and as a consequence the period of the cycle. Phosphorylation/dephosphorylation impacts on the majority of clock proteins (Reischl and Kramer 2011). In particular, the balance of phosphorylation/dephosphorylation of Per proteins by casein kinases (CKs) 1δ and 1ε and protein phosphatase 1 (PP1) appears central in the control of the molecular clock period (Lee et al. 2011): phosphorylation of Per proteins by CK1δ and 1ε regulates the equilibrium between their proteasomal (Akashi et al. 2002; Camacho et al. 2001; Eide et al. 2005) and nuclear addressing (Takano et al. 2004), with dephosphorylation by PP1 stabilizing Per2 and promoting its nuclear accumulation (Gallego et al. 2006; Schmutz et al. 2011). Other kinases such as CK2, glycogen synthase kinase 3β (GSK3β), mitogen-activated protein kinases (MAPK), and adenosine monophosphate (AMP)-activated protein kinase (AMPK) are also important players in determining the stability of

clock proteins and their transfer between nucleus and cytoplasm (Lamia et al. 2009; Weber et al. 2006). Per:Cry complex stability is also under the control of ubiquitination processes mediated by the ubiquitin ligases FBXL21 and SCFFbxl3 which stabilize or promote, respectively, Cry degradation (Hirano et al. 2013). Post-translational SUMOylation (Cardone et al. 2005) and acetylation of Bmal1 and Clock have also been described. In addition to its histone acetyltransferase activity (Doi et al. 2006), Clock acetylates its own partner Bmal1 which enables Cry binding and repression of the Clock-Bmal1 complex (Hirayama et al. 2007). O-GlcNacylation of core clock genes, such as *Clock*, *Bmal1,* and *Per2, i.e.* the transfer of UDP-GlcNac, produced through the hexosamine biosynthetic pathway when glucose levels are elevated, by O-GlcNAc Transferase (OGT), impacts their transcriptional activity and stability (Kaasik et al. 2013; Li et al. 2013). Conversely, the clock protein *Rev-erbα* directly interacts with OGT to regulate its stability and activity (Berthier et al. 2018). All these post-translational events, by controlling clock protein degradation, cellular localization and interaction, constitute an additional layer in the control of clock rhythmicity.

4 Circadian Clock and Metabolism Interplay

4.1 Peripheral Clocks

The molecular machinery described above is present in all organs of the body including liver, adipose tissues (AT), lungs, muscles, etc. collectively called the peripheral clock (Fig. 2). In mice, more than 40% of the protein-encoding genes are

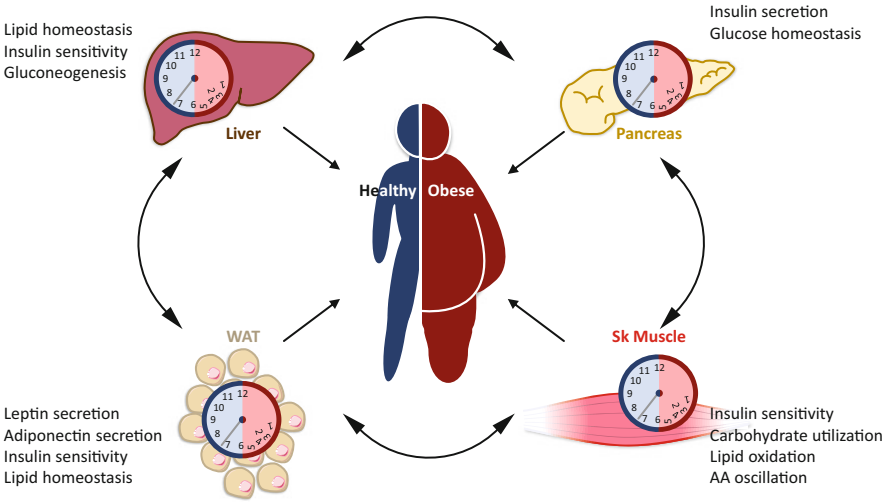

Fig. 2 Peripheral clock cross-talk. Coordinated metabolic processes orchestrated by peripheral clocks are essential to ensure metabolic heath and weight management. In opposition, peripheral clocks desynchronization results in metabolic dysfunctions and obesity

expressed in a circadian manner (Zhang et al. 2014). There is a significant tissue-specificity in the circadian regulation of the transcriptome, which suggests that virtually every gene could be rhythmically expressed in cell types, tissues, or organs. Circadian rhythmicity is not restricted to the transcriptome, but also applies to the acetylome, proteome, phosphoproteome, and metabolome (Dyar et al. 2018a; Mauvoisin et al. 2014). Therefore, although the molecular oscillator is identical throughout the body, the nature, number and phase of circadian gene and protein expression are different from one tissue to another, and thus specific to the function of a given organ. Indeed, peripheral clocks are involved in various physiological functions such as metabolism, signaling, cell division, apoptosis, and DNA repair. Interestingly, energy metabolism is among the most dynamic clock-regulated physiological processes (Panda et al. 2002). In contrast to the central clock, the feeding/fasting cycle constitutes a strong synchronizing signal for peripheral clocks in a number of organs. Indeed, mice fed exclusively during their resting phase (instead of during the active phase) show a progressive phase-shift of the liver clock which adjusts to the new feeding schedule (Damiola et al. 2000; Stokkan et al. 2001), while the central clock remains phase-locked to the day/night alternation. Clocks from other peripheral tissues respond in a variable manner to feeding, some adjusting as rapidly as the liver clock, while others respond more slowly or remain blind to feeding (Damiola et al. 2000; Manella et al. 2021; Stokkan et al. 2001). Interestingly, the liver clock (Wehrens et al. 2017) together with metabolic genes and proteins (Depner et al. 2018) is phase-shifted by restricting food access to a specific time-window also in humans. In both mice and humans, food entrainment of the liver transcriptome and proteome has consequences on body weight and glucose levels (Wang et al. 2017; Wehrens et al. 2017), suggesting that mistimed food intake may contribute to metabolic dysregulation. In sum, while the central clock is essential to define food timing patterns (Challet 2019), food intake strongly impacts the clock in return (Fig. 2).

As mentioned above, not all peripheral clocks behave the same way in response to feeding, suggesting that peripheral clocks may respond to distinct dominant time cues, possibly related to their tissue function. Indeed, day-time feeding exerts a strong effect on liver and white AT (WAT) clocks, leading to a complete inversion of clock genes expression, while the clocks of other tissues such as the kidney and heart were only partially shifted or did not respond to day-time feeding (such as the lung clock) or even lost rhythmicity (as the skeletal muscle clock) (Manella et al. 2021). Interestingly, liver clock disruption in hepatocyte-specific *Bmal1*-deficient mice, while having no effect on the entrainment of other peripheral organ clocks by feeding (the phase of clock genes were comparable in WAT of hepatocyte-specific *Bmal1* deficient and control mice, for instance), skewed the WAT transcriptome toward a complete inversion due to the emergence of new subsets of rhythmic genes that were strongly shifted by day-time feeding (Manella et al. 2021). These data emphasize the importance of the liver clock in influencing the response of peripheral tissues to feeding irrespective of their clocks.

4.2 Circadian Clock and Nutrient Sensing

Clock synchronization by feeding requires that the biological clock senses nutrients and metabolites. Multiple interactions have been identified between clock genes and intracellular metabolic sensors, including cellular redox level. The nicotinamide adenine dinucleotide (NAD+) /NADH ratio, which reflects the energy status, tightly associates with the feeding/fasting cycle. It has been shown that Bmal1 and Clock directly activate the transcription of the nicotinamide phosphoribosyltransferase (NAMPT) gene encoding the rate-limiting enzyme of the NAD salvage synthesis pathway, leading to circadian oscillations in NAD+ levels. In turn, Sirtuin 1 (SIRT1), a deacetylase which requires NAD+ as cofactor, regulates the circadian machinery by modulating the acetylation levels of Bmal1 and Per2 (Asher et al. 2008; Nakahata et al. 2008). PARP-1, a poly adenosine diphosphate (ADP) Ribose polymerase 1, which also uses NAD+ as cofactor, presents circadian variations in its activity and induces circadian poly ADP ribosylation of Clock in mouse liver, hence altering its ability to bind DNA (Asher et al. 2010). In addition, it has been described that SIRT1 counteracts histone acetyltransferase activity of Clock. Indeed, SIRT1 by physically interacting with Clock modulates the acetylated level of Clock targets thereby linking metabolic status to chromatin modifications (Nakahata et al. 2008).

AMPK, another key intracellular metabolic factor which senses the AMP/Adenosine triphosphate (ATP) ratio, also interacts with clock components. On the one hand, AMPK subunit composition, subcellular localization as well as the phosphorylation of the AMPK substrates Raptor and acetyl coenzyme A carboxylase 1 (ACC1) display circadian oscillations in mouse liver (Lamia et al. 2009). On the other hand, AMPK acts on the circadian clock by mediating Cry1 and Per2 phosphorylation, which leads to their degradation (Lamia et al. 2009; Um et al. 2007).

The activity of mTOR, another important nutrient sensor related to the insulin signaling pathway, is also time-regulated and in turn controls Bmal1, Clock, and Cry1 expression (Ramanathan et al. 2018).

PPARs (peroxisome proliferator-activated receptors), which are nuclear receptors activated by fatty acids, also interplay with the biological clock. The expression of PPARα, which controls many genes involved in lipid metabolism and energy homeostasis, exhibits circadian variations in the liver as well as in white and brown adipose tissues (Lemberger et al. 1996; Yang et al. 2006). PPARα, on its turn, directly regulates *Bmal1* transcription (Canaple et al. 2006) and modulates Per2 activity (Schmutz et al. 2010). PPARα has thus been identified as a key transducer of peripheral clock synchronization by food intake timing (Mukherji et al. 2015a). PPARγ gene expression also oscillates in WAT and liver (Yang et al. 2006). Interestingly, while High-Fat-Diet (HFD)-feeding induces alterations in the expression and diurnal oscillations of clock genes and nuclear receptors, including PPARγ (Kohsaka et al. 2007), PPARγ plays a role in HFD-induced clock reprogramming (Eckel-Mahan et al. 2013). In addition, the clock gene *Per2* regulates lipid metabolism by directly repressing P*PARγ* transcriptional activity (Grimaldi et al. 2010). Moreover, liver PPARδ controls the diurnal plasma lipid profile, thereby controlling muscle PPARα-regulated fatty acid use. PPARδ and PPARα coordinate liver

lipogenesis and muscle fatty acid uptake. Altogether, these data indicate that PPARs regulate not only the energy sensing by the clock, but also the metabolic cross-talk between peripheral tissues.

Beside these signaling pathways, most metabolites such as glucose, lipids, and amino acids show daily variations in blood and tissues, and may thus also contribute in proper synchronization of the different peripheral clocks. Indeed, metabolite levels oscillate in a tissue-specific manner, while also exhibiting temporal correlations across tissues (Dyar et al. 2018a). In addition, the expression of metabolite transporters, such as GLUT4 and GLUT2 which are responsible for muscle and liver glucose uptake respectively, is also clock-driven (Dyar et al. 2014; Lamia et al. 2008), hence enabling an optimal communication between metabolites and their carriers. Interestingly, *Clock* and *Bmal1* disruption in liver and muscle, as well as HFD feeding, results in aberrant or dampened circadian oscillations in metabolites, bile acids, and nucleotide levels, which may contribute to metabolic disturbances (Dyar et al. 2018a, b; Eckel-Mahan et al. 2013; Fustin et al. 2012). In line, liver Bmal1 rescue in whole-body *Bmal1*-deficient mice enabled an evaluation of the independence of the liver clock from other peripheral clocks (Koronowski et al. 2019). Remarkably, while the liver clock is fully functional in this model, only 10% and 19% of normally rhythmic genes and metabolites, respectively, are still cycling, suggesting that while the liver clock displays autonomous metabolic functions, such as glycogen and NAD+ metabolism, integration of external signals is essential, notably for proper lipid metabolism (Koronowski et al. 2019). Altogether, these studies shed light on the essential role of the inter-organ communication of peripheral clocks in maintaining tissue rhythmicity and function (Fig. 2).

Moreover, hepatocyte-specific deletion of both *Rev-erbα* and *Rev-erbβ* strongly impacts the circadian transcriptome and metabolism not only in hepatocytes, but also in non-hepatocytic liver cells. Interestingly, loss of *Rev-erbs* in hepatocytes induces a cell type specific transcriptome remodeling with little overlap between endothelial cells, Kupffer cells, and hepatocytes (Guan et al. 2020). This study highlights the importance of paracrine cell communication, likely through metabolites, in the control of energy homeostasis by peripheral clocks.

In conclusion, nutrient sensing by peripheral clocks is essential to gate metabolic processes to the optimal time-window when integrating environmental cues (i.e., feeding time) and by coordinating metabolomes and transcriptomes locally and among tissues to maintain whole-body energy balance.

4.3 Circadian Regulation of Hormones

Rhythmic oscillations in circulating hormones are also an essential feature of the interplay between the clock and the metabolic status. Glucocorticoids (GC), cortisol in humans or corticosterone in mice, are steroid hormones produced by the adrenal glands exerting important metabolic actions. Cortisol exhibits circadian oscillations characterized by a morning peak and lowest concentrations during the night/sleep phase in humans. In the absence of stress which acutely or chronically stimulates

glucocorticoid secretion, the daily cortisol rhythm can be used to evaluate the period of the clock in humans. GC are a potent synchronizing signal for peripheral clocks (Balsalobre et al. 2000). In SCN-ablated mice, a single activation of the glucocorticoid receptor (GR) synchronizes 60% of the liver circadian transcriptome, an effect mediated by HNF4 (Reddy et al. 2007). Interestingly, GC inhibit liver clock resetting by feeding only when the timing of food intake conflicts with the endogenous phase (i.e., during the rest phase), suggesting that the central clock may use GC to prevent the uncoupling of peripheral clocks (Le Minh et al. 2001). GC has been shown to repress Rev-erbα liver expression in a dose-dependent manner (Torra et al. 2000). In addition, GR and Rev-erbα functionally interact through HSP90, hence reciprocally modulating their stability and subcellular localization (Okabe et al. 2016). From a functional point of view, this interaction mediates time-dependent GC action on carbohydrate and lipid metabolism in mouse livers (Caratti et al. 2018).

The daily variations of plasma insulin levels are profoundly affected by meals, which induce a post-prandial release of insulin (Tasaka et al. 1980). In response to the same amount of glucose, insulin secretion is higher but shorter in the morning and lower and longer in the evening, demonstrating diurnal variations in insulin sensitivity (Van Cauter et al. 1991). A recent study in mice showed that insulin and lGF-1 are major players in the synchronization of the circadian clock by food intake by driving Period protein expression (Crosby et al. 2019). In line, diabetic mice exhibit altered clock gene expression (Shostak et al. 2013). Mechanistically, insulin directly regulates Bmal1 nuclear localization and transcriptional activity through post-prandial Akt-mediated Bmal1 phosphorylation (Dang et al. 2016). Plasma glucagon exhibits diurnal variations with higher concentrations during the inactive phase in rodents and humans which also coincides with fasting (Ruiter et al. 2003; Tasaka et al. 1980). In addition, in vitro mixed human islet-cells display circadian rhythmicity in insulin and glucagon secretion which is lost in T2D islet cells (Petrenko et al. 2020). In a model of lung-associated cachexia, glucagon promotes proteasomal degradation of hepatic Rev-erbα through cAMP/PKA signaling, resulting in elevated hepatic glucose production (Verlande et al. 2021). Altogether, these studies demonstrate that a tight interaction between hormone secretion and the biological clock is essential to control energy balance in response to altered feeding/fasting cycles.

5 Circadian Control of Energy Homeostasis and Metabolic Consequences of Clock Disruption

5.1 Adipose Tissue Clock and Obesity

Organisms require energy supply across the day/night cycle, even though nutrient intake is diurnal. The balance between energy storage and production, which controls energy homeostasis, is tightly regulated by the biological clock. Consequently, clock misalignment results in major metabolic alterations, which have been extensively studied in animal models. Indeed, whole body or tissue-specific clock

gene deletion leads to profound metabolic perturbations. WAT is an important metabolic and endocrine tissue essential for lipid storage. Dysregulations of WAT lipid metabolism are implicated in obesity development. Bmal1 and Clock regulate diurnal variations in WAT lipolysis rate, plasma free fatty acid (FFA), and glycerol levels through direct control of the expression of the *Atgl* and *Hsl* genes encoding key lipolytic enzymes (Shostak et al. 2013). As a result, *Clock Δ19* mutant mice and *Bmal1*-deficient mice display reduced FFA and glycerol levels, especially in response to fasting (Shostak et al. 2013). In addition, HFD-fed *Clock Δ19* mutant mice exhibit exacerbated adipocyte hypertrophy as well as elevated plasma leptin during the light phase (Turek et al. 2005), accompanied by hypoinsulinemia and hyperglycemia. In line with a role of the clock in adipose tissue fatty acid storage and mobilization, global *Rev-erb*α-deficient mice were shown to display increased adiposity on chow diet and higher susceptibility to HFD-induced obesity (Delezie et al. 2012). More recently, it has been shown that adipocyte-specific *Rev-erbα*-deficiency had a limited impact on fat mass and no changes in WAT lipogenic gene expression in mice fed a chow diet. However, when fed a HFD, adipocyte-specific *Rev-erbα*-deficient mice were more prone to obesity while being protected from insulin resistance (Hunter et al. 2021). WAT-specific *Bmal1* deletion results in a blunted rhythmic expression of clock genes as well as altered feeding behaviors, thus promoting obesity. Interestingly, the modification of feeding rhythms in WAT-specific *Bmal1* deficient mice results from a lower release of polyunsaturated fatty acids (PUFAs) by adipocytes which directly control the feeding pattern by regulating hypothalamic neuropeptide expression (Paschos et al. 2012). Obesity is characterized by the accumulation of visceral fat associated with inflammation. Interestingly, alterations in circadian gene expression are associated with exacerbated inflammation in omental adipocytes from obese humans. These circadian clock and inflammatory dysfunctions are related to NF-KB over-activation, which results in modifications of *Bmal1*-mediated target gene transcription by promoting Bmal1 chromatin relocalization (Maury et al. 2021).

Interestingly, HFD-fed mice exhibit altered feeding patterns and circadian locomotor activity as well as changes in clock gene expression in peripheral tissues as well as in the hypothalamus (Kohsaka et al. 2007) (Fig. 3). While chow-fed mice consume most (approx. 80%) of their food during the active phase, food intake during the inactive/sleep phase increases significantly upon HFD feeding, which is paralleled by increased body weight and deteriorated metabolic parameters (Kohsaka et al. 2007). This suggests that, beside the amount of ingested calories, mistimed feeding may entrain the clocks at the "wrong" time, further amplifying a vicious circle leading to weight gain and altered glucose/lipid homeostasis. WAT also contributes to energy homeostasis through the release of adipokines such as leptin. Leptin is an anorexigenic hormone communicating energy level to the central nervous system and whose secretion is increased by meal ingestion and obesity, and decreased by fasting (Bodosi et al. 2004). Although obese subjects exhibit higher levels of leptin, they are less sensitive to leptin, which exacerbates weight gain. Leptin secretion displays circadian oscillations both in humans and rodents (Saladin et al. 1995), with higher levels during the night in humans (Licinio et al. 1997).

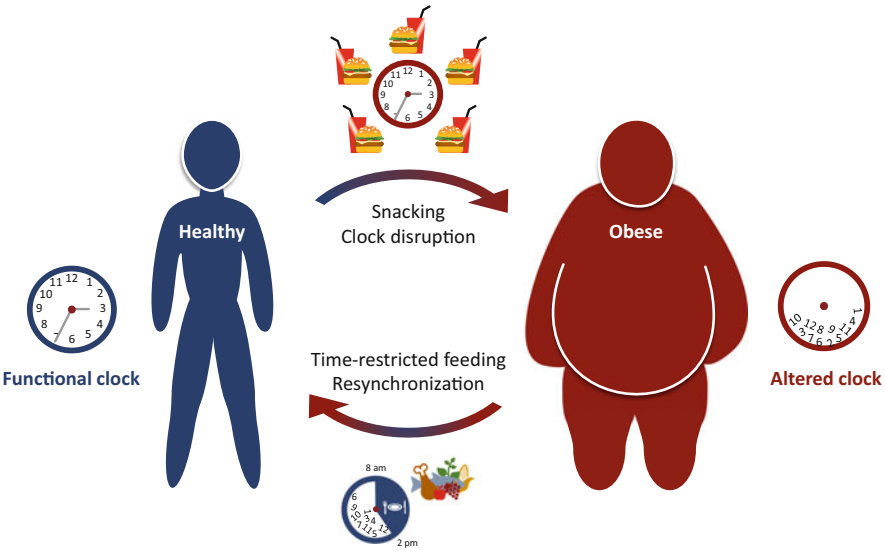

Fig. 3 Food habits alteration and time-restricted feeding in obesity. Snacking and high caloric food intake promotes clock alteration and obesity. On the other hand, time-restricted feeding and low caloric intake allow to resynchronize clock and would favor to decrease obesity

Interestingly, mice subjected to a chronic jet lag display arrhythmic leptin secretion associated with central leptin resistance and fat weight gain (Kettner et al. 2015). Moreover, deletion of *Rev-erbα* and *β* in hypothalamic nuclei outside the SCN of male (but not female) mice exacerbates HFD-induced weight gain by regulating diurnal leptin sensitivity in the arcuate nucleus (Adlanmerini et al. 2021). Thus, leptin resistance induced by clock misalignment might play an important role in clock disruption-associated obesity. Adiponectin, another major adipokine implicated in peripheral energy state sensing, is also clock-regulated. Circulating adiponectin levels exhibit diurnal oscillations in humans (Gavrila et al. 2003). In addition, gene expression of adiponectin and its receptor is circadian in human adipose tissue depots (Gómez-Abellán et al. 2010). Strikingly, adiponectin controls feeding rhythms and appetite through adiponectin receptor-mediated *Bmal1* induction in hypothalamic neurons (Tsang et al. 2020). Additionally, adipokines act on non-adipose tissue metabolism. Indeed, leptin, by directly stimulating AMPK alpha 2 subunit phosphorylation in skeletal muscle, inhibits acetyl-CoA carboxylase (ACC) activity which promotes muscle fatty acid beta-oxidation (Yamauchi et al. 2001). Adiponectin enhances insulin sensitivity by decreasing muscle triglyceride content through the activation of beta-oxidation (Minokoshi et al. 2002; Yamauchi et al. 2001). Overall, clock dysfunction in WAT will not only lead to altered lipid metabolism, but also defective communications between the peripheral and central systems, hence exacerbating metabolic disorders.

5.2 Circadian Glucose Homeostasis and Diet-Induced Obesity-Associated Metabolic Disorders

The pancreas plays also a critical role in glucose metabolism through insulin and glucagon production. In addition to feeding/fasting cycles, pancreatic hormone production is also controlled by an intrinsic pancreatic biological clock. Indeed, murine pancreatic islets exhibit circadian oscillations of clock gene expression, which are abolished in *clock* mutant mice (Marcheva et al. 2010). In addition, islets isolated from *clock* mutant mice display reduced insulin secretion in response to glucose, likely due to increased islet apoptosis and decreased islet proliferation (Marcheva et al. 2010). Similar defects were observed in mice specifically depleted for *Bmal1* in β-cells, i.e. hyperglycemia and glucose intolerance due to altered insulin secretion (Marcheva et al. 2010; Perelis et al. 2015). Consistent with these studies, conditional β-cell-specific Bmal1 overexpression improves glucose-stimulated insulin secretion and overcomes HFD-induced glucose intolerance (Rakshit and Matveyenko 2021). Interestingly, in vitro treatment of mice and human islets with nobiletin, a ROR agonist which has been described to protect mice from HFD-induced metabolic dysregulation in a clock-dependent manner (He et al. 2016), increased glucose-stimulated insulin secretion (Rakshit and Matveyenko 2021). From a mechanistical perspective, it has been elegantly demonstrated that Bmal1 and Clock control the expression of genes involved in insulin exocytosis through the regulation of alternative mRNA splicing (Marcheva et al. 2020).

Skeletal muscle is critical for whole-body homeostasis as it represents approximatively 40% of body weight and handles up to 80% of post-prandial glucose. Skeletal muscle function is profoundly affected by obesity, notably due to a myofiber type switch and intramyocellular lipid accumulation, which exacerbate obesity-associated glucose mishandling. Similar as other key metabolic tissues, skeletal muscle harbors an intrinsic biological clock synchronized by physical activity and feeding patterns (Wolff and Esser 2012; Yamanaka et al. 2008). The muscle clock is essential in controlling diurnal metabolism and orchestrates the daily shift from carbohydrate utilization to lipid oxidation which temporally gates the expression of genes implicated in substrate utilization and storage (Hodge et al. 2015). As an example, muscle-specific *Bmal1* deletion reduces muscle insulin sensitivity, leading to decreased muscle glucose uptake associated with a defect in GLUT4 translocation (Dyar et al. 2014; Harfmann et al. 2016). Interestingly, the muscle clock also controls protein degradation and synthesis. *Rev-erbα*-deficient mice display reduced fiber size associated with increased expression of atrophy-related genes. On the contrary, pharmacological Rev-erb activation blunts dexamethasone-induced muscle atrophy (Mayeuf-Louchart et al. 2017). Additionally, *Rev-erbα* controls skeletal muscle mitochondrial biogenesis and oxidative function by regulating Ampk-Sirt1-Ppargc1α signaling. As a consequence, *Rev-erbα*-deficiency leads to reduced skeletal muscle mitochondrial content and oxidative function, resulting in compromised exercise capacity (Woldt et al. 2013). Similarly, mitochondrial respiration of human skeletal muscle biopsies showed

strong diurnal rhythmicity with a peak during the late evening. This was driven by intrinsic changes in mitochondrial oxidative capacity rather than modification of the mitochondrial content (van Moorsel et al. 2016). Interestingly, this diurnal variation in mitochondrial respiration, along with *Per 2* gene expression rhythmicity, is lost in older, overweight, and glucose-intolerant patients (Wefers et al. 2020). In line, skeletal muscle expression of the clock genes *Cry1, Cry2, Dbp* and *Clock* is compromised in obese men, whereas only *Cry1* and *Dbp* gene expression is altered in obese women (Sardon Puig et al. 2020).

5.3 The Liver Clock, Lipid Metabolism, and Obesity-Associated Liver Disease

Dysregulation of hepatic metabolism also strongly correlates with obesity. Non-alcoholic fatty liver disease (NAFLD) represents today one of the most common liver pathologies predisposing to liver transplantation in the most severe cases. With a prevalence of approximately 25% of the world population, NAFLD is evolving in conjunction with the obesity epidemic (Younossi et al. 2016). Indeed, it has been estimated that 80% of obese people are likely to develop NAFLD (Milić et al. 2014). NAFLD pathogenesis is complex, progressing from isolated steatosis to non-alcoholic steatohepatitis (NASH), fibrosis, and cirrhosis. Interestingly, animal models with impaired clock function exhibit also hepatic lipid metabolism disturbances. *Clock* mutant as well as liver-specific *Rev-erb*-deficient mice develop steatosis associated with elevated triglyceride levels (Bugge et al. 2012; Guan et al. 2020; Turek et al. 2005). Elevated serum triglycerides are also observed in single *Rev-erb*α deficient mice (Raspé et al. 2002). Additionally, liver-specific expression of a DNA-binding domain deficient *Rev-erb*α mutant, in combination with hepatocyte-specific deletion of *Rev-erb*β, impacts on the circadian expression of core clock genes, with deleterious consequences on circulating glucose and triglyceride levels (Cho et al. 2012). Mechanistic studies using mice harboring this mutated *Rev-erb*α form suggested that Rev-erbα regulates hepatic lipid metabolism-related genes, such as lipoprotein lipase, CD36, and fatty acid synthase, via its tethering to HNF6 (Zhang et al. 2015, 2016). In contrast with these data, a recent study highlighted that hepatocyte-specific deletion of *Rev-erb*α has limited impact on lipid metabolism in steady-state conditions (Hunter et al. 2020). Interestingly, this study suggests that the role of Rev-erbα is unveiled only upon 24 h fasting followed by mistimed feeding (Hunter et al. 2020). Mitochondrial dysfunction induced by nutrient excess during obesity also strongly impacts on NAFLD progression (de Mello et al. 2018; Mukherji et al. 2019). Several studies have evidenced that mitochondrial function, dynamics, and content exhibit circadian oscillations (Neufeld-Cohen et al. 2016; Peek et al. 2013; Schmitt et al. 2018). Indeed, 24-h variations are observed in FA oxidation (FAO) in liver homogenates with higher rate at the end of the resting period. Interestingly, diurnal oscillations in FAO enzymes as well as mitochondrial respiration are blunted in *Per1/2* deficient mice (Neufeld-Cohen et al. 2016). Likewise, liver-specific *Bmal1*-deficient mice exhibit impaired

FAO and mitochondrial respiratory capacity related to NAD+ deficiency (Peek et al. 2013). In addition, the regulation of mitochondrial dynamics by the biological clock occurs through the circadian regulation of Dynamin-related protein 1 (DRP1) phosphorylation which controls mitochondrial fusion and fission cycles (Schmitt et al. 2018). In line, Bmal1 also regulates liver mitochondrial dynamics and metabolic flexibility in accordance with fasting/feeding cycles. As a consequence, liver-specific *Bmal1* deletion results in enlarged and swollen mitochondria associated with hepatic steatosis (Jacobi et al. 2015).

Dysregulated glucose metabolism often occurs in association with NAFLD and obesity as well as upon clock function alteration. In addition to altered lipid metabolism, *Clock* mutant mice exhibit profound metabolic disturbances, including hyperglycemia and hypoinsulinemia (Turek et al. 2005). *Clock* mutant and *Bmal1*-deficient mice exhibit a strong hypoglycemic response to insulin due to decreased gluconeogenesis although the corticosterone and glucagon feedback responses are preserved. This phenotype is associated with blunted circadian oscillations in liver PEPCK activity in *Clock* mutant mice (Rudic et al. 2004). Liver-specific Cry-overexpression in *db/db* mice decreases blood glucose and improves insulin sensitivity by repressing glucagon-mediated PKA phosphorylation of CREB and gluconeogenic gene expression during fasting (Zhang et al. 2010). In addition, Cry1 and Cry2 block GR-mediated induction of *Pepck* gene expression (Lamia et al. 2011).

A number of studies indicate a deleterious role of circadian clock disruption in mouse models of NASH and fibrosis. For instance, *Per2*-deficiency exacerbates carbon tetrachloride (CCl4)-induced fibrosis in mice. This phenotype is associated with increased activation and decreased apoptosis of hepatic stellate cells (Chen et al. 2010). Likewise, *PPARα*-deficient mice develop higher levels of fibrosis associated with elevated intrahepatic triglycerides when fed a methionine and choline deficient diet (MCD) (Emilia et al. 2003). Strikingly, chronic jet lag in mice accelerates steatosis, inflammation, and hepatic fibrosis, even leading to spontaneous hepatocellular carcinoma after 90 weeks of chronic jetlag. This was associated with a global metabolic dysregulation and elevated liver damage makers, such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST) (Kettner et al. 2016).

Taken together, these data demonstrate that peripheral biological clocks are intimately connected and coordinately orchestrate tissue metabolism to ensure whole-body energy homeostasis and body weight maintenance.

6 Clock Desynchronization and Metabolic Syndrome in Humans

Our modern way of life has dramatically impacted on biological rhythms. Physical activity, food intake, and light exposure are no longer limited to daylight hours (i.e., natural light), and sleep duration has been drastically reduced. Due to the economic and societal pressure, around 20% of the working population are shift workers (Kivimäki et al. 2011) who expose themselves to light sources during the night,

thereby disrupting the central clock. This is accompanied by a disruption of usual sleep patterns with access to food that occurs during atypical hours. The complex interaction of these different components has harmful effects on the body, as shown by an increased risk of developing cancer, sleep disorders or even metabolic diseases among shift workers compared to day-time workers (Drake et al. 2004; Garaulet et al. 2010; Masri and Sassone-Corsi 2018). Indeed, observational and meta-analysis studies showed that shift workers, compared to day-time workers, are more likely to develop the metabolic syndrome, obesity, type 2 diabetes, and hypertriglyceridemia (Karlsson et al. 2001; Osaki et al. 2021; Pietroiusti et al. 2010; Gan et al. 2015; Gao et al. 2020). In addition, shift workers develop abnormally high levels of glycated hemoglobin (HbA1c), as well as altered expression of peripheral clock genes, already at a young age (Rizza et al. 2021). Assessing blood mononuclear cell gene expression profiles in night workers identified increased endoplasmic reticulum (ER) stress, decreased oxidative stress regulators (such as Nrf2) together with a deregulation of the clock as possible underlying mechanism responsible for the adverse metabolic changes (Ferraz-Bannitz et al. 2021). Chronic activation of oxidative stress and endoplasmic reticulum are indeed involved in the pathogenesis of the metabolic syndrome (Marseglia et al. 2014). In addition, the quality and duration of sleep are strongly impacted in shift workers, and several studies have highlighted the harmful effects of sleep deprivation on metabolism (Broussard and Van Cauter 2016; Lim et al. 2018; Spiegel et al. 2009). Finally, a recent meta-analysis shows once again the close correlation between shift work and the increased risk for metabolic diseases. Indeed, interventions in shift workers, such as rapid rotation shifts or wearing light-blocking glasses, can have beneficial effects on BMI and blood pressure (Crowther et al. 2021).

Besides shift work, exposure to blue light and/or social activity may lead to clock misalignment, a phenomenon coined "social jet lag." Indeed, these individuals adopt different rhythms between week days, "days on," and the weekend or "days off" (Hatori et al. 2017; Roenneberg et al. 2012). Modeling combination of social jet lag associated with a cafeteria-type diet is sufficient to induce features of the metabolic syndrome in rats (Espitia-Bautista et al. 2017).

7 Pathological Consequences of Altered Feeding Patterns and Potential Beneficial Impact of Time-Restricted Feeding/Eating to Decrease Obesity and Improve Metabolic Health

7.1 Feeding Patterns and Impact of Time-Restricted Feeding (TRF) on Metabolic Control in Rodents

As mentioned above, feeding acts as a dominant time cue for circadian clocks in metabolic organs. Limiting food access to the resting/sleep period in rodents, which are nocturnal animals normally consuming most of their food during the active/night phase, entrained not only peripheral clocks in metabolic organs (Le Minh et al. 2001;

Stokkan et al. 2001; Vollmers et al. 2009), but also altered clock-driven metabolic gene expression to an inappropriate time, resulting in increased body weight and fat mass (Salgado-Delgado et al. 2010; Wang et al. 2017). In line, time-restricted feeding (TRF) to the rest period in standard diet-fed rats increases adiposity despite a decreased caloric intake (Ramirez-Plascencia et al. 2017). Interestingly, when given ad libitum access to a HFD, mice consume a higher percentage of their daily food intake during the rest period, which was found associated with weight gain (Kohsaka et al. 2007), suggesting that the timing of food intake is important for metabolic fitness. This seminal finding was confirmed by numerous reports. Indeed, rodents consuming the same amount of calories, but during the resting period (i.e., at the "wrong circadian time") display an exacerbated weight gain (Arble et al. 2009; Mukherji et al. 2015b; Sundaram and Yan 2016; Yasumoto et al. 2016). By contrast, consuming the same amount of calories exclusively during the active/normal feeding phase significantly reduces or prevents weight gain and adiposity (Bray et al. 2010; Chaix et al. 2014; Hatori et al. 2012) (Fig. 3). This response was associated with improved lipid metabolism, ameliorated insulin sensitivity and reduced hyperglycemia, inflammation (Chaix et al. 2014; Zarrinpar et al. 2014), and liver steatosis (Hatori et al. 2012), due, at least in part, to the restoration of strong oscillations of metabolic sensors and regulators, such as CREB, mTOR, and AMPK (Hatori et al. 2012). TRF of Drosophila to a 12-h window counteracted the onset of obesity and obesity-related hypertriglyceridemia and insulin resistance and protected from muscle lipid deposition, thereby improving muscle function (Villanueva et al. 2019). Interestingly, feeding mice a HFD resulted in weight gain, regardless of the number of calories ingested, unless access to the HFD was limited to a time window as short as 4 h (and hence a very long fasting period) resulting in reduced weight gain compared to control mice fed an ad libitum standard or high fat diet (Sherman et al. 2012). Strikingly, AMPK and SIRT1 being increased in this study, fasting duration might be important for the beneficial effect of time-restricted feeding by imposing a clear separation of the catabolism and anabolism cycles (Chaix et al. 2014; Hatori et al. 2012). This dietary approach may be instrumental not only for prevention of weight gain, but also to reverse diet-induced obesity and associated disorders. Several studies showed that imposing TRF in mice with genetically altered clocks consolidates dietary/metabolic rhythms and partially restores hepatic gene rhythmicity, and may even reduce body weight and fat mass, inflammation, plasma lipids, and, depending on the genetic models, glucose tolerance (Adamovich et al. 2014; Chaix et al. 2019; Greenwell et al. 2019; Vollmers et al. 2009). In HFD-induced obese mice, 9 h TRF during the active phase reversed diet-induced obesity and metabolic derangements (Chaix et al. 2014). It is noteworthy that TRF had no beneficial effect when mice were fed a high fructose diet except for a reduction in fat mass (Chaix et al. 2014). Together these data suggest that the timing of food intake, regardless of the amount of calories ingested, may lower body weight gain both when feeding high fat as well as standard diets. Finally, while most studies indicate that food intake is not significantly decreased in TRF, the amount of calories may be slightly lower in TRF, which may have contributed to weight reduction over time.

7.2 Feeding Pattern and Metabolic Control in Humans: Potential Beneficial Impact of Time-Restricted Feeding/Eating to Decrease Obesity and Improve Metabolic Health

In humans, mistimed food intake may also contribute to metabolic dysregulation and the global rise in obesity. Rhythms of living, hence food intake patterns, have evolved tremendously over the last century: 24/7 access to calorie-dense food has led to extended caloric intake over the 24 h period in the general population. When recording the timing of food intake in healthy young adults, it was found that >50% spread their food/caloric intake over at least 15 h, from 6 a.m. to 11 p.m., with less than 25% of their daily calorie intake ingested before noon (Gill and Panda 2015). Thus, meal timing is no more fully aligned with the active/light phase, which may result in adverse metabolic adaptations. As in rodents, feeding is an important time cue, and food intake at unusual times of the day induces a phase-shift in the plasma proteome (Depner et al. 2018), with consequences on glucose levels (Wehrens et al. 2017). In line, desynchronization of healthy young adults in well-controlled conditions (reversal of activity and food intake at night), while maintaining an isocaloric food intake, rapidly reduces systemic and muscle glucose tolerance and increases blood pressure (Scheer et al. 2009; Wefers et al. 2018). Thus, the question arising from these observations is whether imposing a time-restricted eating (TRE) protocol may prevent the development of obesity and metabolic imbalance and may even normalize metabolic parameters also in humans. To answer this question, the effect of a time-restricted eating protocol, by limiting access to a time window of 10 h/day, was assessed for 16 weeks in 8 overweight subjects, without any other instruction regarding their caloric or macronutrient intake. The subjects (initial BMI of approx. 33) lost on average 3.3 kg body weight after 16 weeks (Gill and Panda 2015). It is worth noting that the estimated caloric intake during the 16-week intervention period was 20% lower than during the pre-intervention period (Fig. 3). Interestingly, all participants slept better and felt more energetic, and voluntarily continued with the 10–11 h TRE after the completion of the 16-week intervention. After 36 weeks (1 year since the intervention began), the participants maintained weight loss and sleep improvement (Gill and Panda 2015). A more recent study tested the effect of restricting the feeding window to 8 h/day (10 a.m. – 6 p.m.) for 12 weeks in obese subjects (BMI approx. 35), which resulted in a 2.6% weight reduction, a reduction in systolic (but not diastolic) blood pressure, and reduced fat mass (Gabel et al. 2018). In addition, reducing food access by an average of 4 h down to 10 h for 12 weeks in patients with features of the metabolic syndrome significantly reduced body weight and abdominal fat mass and reduced blood pressure (Wilkinson et al. 2020). In another study, TRE to 9 h for 7 days improved fasting glucose, post-prandial glycemia and insulinemia in overweight subjects (Hutchison et al. 2019). In contrast, other studies found little or no effects of TRE in humans (Carlson et al. 2007; Stote et al. 2007), possibly due to the fact that the temporal window of food access was shifted to late in the day rather than in the morning. This was tested in a cross-over study comparing the effect over 5 weeks of a 12 h window (7 a.m. – 7 p.m.) with a restricted window limited to the first part of

the day (early TRE: 7 a.m. – 1 p.m.), divided over three meals in both conditions. The overweight (BMI 32) and pre-diabetic subjects consumed the same amount of calories in the two study arms (Sutton et al. 2018). There was no difference in fasting blood or OGTT glucose, circulating lipid levels, or markers of inflammation. In contrast, reduction of the morning hours window (early TRE) significantly reduced fasting plasma insulin levels, improved β-cell function as measured by the insulinogenic index, improved insulin sensitivity, and reduced blood pressure. These effects were observed when the protocol was designed to maintain body weight (eucaloric diet). In contrast, in a cross-over study in healthy adults consuming a normal diet consisting of either three meals distributed over the day or one meal provided in the early evening with an equal total calorie intake, restricting food access to the end of the day for 8 weeks did not significantly reduce body weight, blood glucose, and lipid levels (Stote et al. 2007). However, subjects reported hunger feelings, and the drop-out rate was high (>30%). A similar study reported no effect on body weight, and an increase in fasting glucose and glucose intolerance, suggesting that restricting food intake to the end of the day has no effect, or may even have adverse effects on glycemic control (Carlson et al. 2007). In line, late food intake was associated with reduced energy expenditure and decreased glucose tolerance (Bandín et al. 2015). Finally, several reports indicate greater weight loss when calories were ingested in the first part of the day in overweight subjects (Garaulet et al. 2013; Jakubowicz et al. 2013) or diabetic patients (Jakubowicz et al. 2015). Altogether these data suggest that besides the amount of calories, the time of food intake is an important feature to integrate in weight management and metabolic improvement protocols (Fig. 3). However, further studies comparing diets with different composition and duration and with larger numbers of recruited subjects with known chronotypes, and more detailed calorie intake measurements, are still required.

8 Conclusion/Perspectives

Our modern way of life has strongly impacted the biological rhythms of our bodies and this contributes to the surge of the obesity pandemic. As discussed in this chapter, the use of genetically clock-disrupted animal models has largely enabled the understanding of how the biological clock interacts with metabolism. Nutrient sensing by the clock, but also peripheral clock inter-organ communication are essential to coordinate the rhythmicity of metabolic processes, which is fundamental to maintain the whole-body energy balance in response to feeding/fasting cycles. As a consequence, alterations in the biological clock generated by shift working, for example, result in profound metabolic dysregulations arising from mistimed feeding patterns and the ensuing desynchronization between the central and peripheral clocks. The last decades have raised the interest in TRF as a potential and beneficial way to decrease obesity and improve metabolic health. It appears now clear that, beyond the diet composition, food timing is a crucial element to be taken into consideration to maintain proper weight control. Notwithstanding, further clinical

studies are necessary to connect molecular clock alterations to metabolic dysfunction with the aim of developing new dietary and/or therapeutic interventions to improve metabolic health in clock-disrupted patients.

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Nutrition and Microbiome

Nathalie M. Delzenne and Julie Rodriguez

Contents

Abstract

The prevalence of overweight and obesity has reached epidemic proportions globally over the past few decades. The search for new management approaches continues and among them, targeting the gut microbiota can be envisioned. To date, numerous data showed the involvement of the gut microbes in the regulation and control of host metabolism. There are also increasing evidences highlighting the interactions between environmental factors, intrinsic factors, gut microbiota, and metabolic diseases. Diet emerges as the most relevant factor influencing the gut microbiome. Eating habits, as well as short-term consumption of specific

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diets, alter the gut microbiota composition. Moreover, nutritional disorders are associated with changes of the gut microbiota composition and/or function, as shown in obesity or type 2 diabetic patients versus healthy lean subjects. Targeting the gut microbiota for improving metabolic health appears as a new approach to manage obesity and cardio-metabolic risk. In this review, we have detailed the results of human interventions targeting the gut microbiome by prebiotic supplementation, prebiotics being defined as "substrates that are selectively utilized by the host microorganisms conferring a health benefit." If the potential benefit of this approach is obvious in preclinical models, the efficacy of prebiotics in humans is less reproducible. The inter-individual variability of response to dietary intervention can be dependent on the gut microbiota and we summarized the basal gut microbiota characteristics driving the metabolic response to dieting, prebiotic and dietary fiber intervention in the context of obesity and related metabolic diseases.

Keywords

Gut microbiota · Metabolic health · Nutrition · Prebiotics

1 Introducing the Role of the Microbiome for Human Health

1.1 The Definition of Microbiome

In the last decade, the role of microbiome in health has gained interest in science, medicine, and more generally for a broad audience. Key scientific outcomes highlight the link between the microbiome and the regulation of host physiological functions including the regulation of immunity, appetite, metabolism, and behavior (Zheng et al. 2020). In order to standardize the emergent studies focusing on the microbiome, a panel of experts recently revisited the microbiome definition (Berg et al. 2020). In this new definition, the microbiome encompasses both the microorganisms, including viruses, bacteria, archaea, unicellular eukaryotes, and fungi, and their "theater of activity" (structural elements, metabolites/signal molecules, and the surrounding environmental conditions). The microbiome probably comprises thousands of different bacterial species, the highest density of bacteria being present in the large intestine.

The dominant gut microbial phyla are Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, and Verrucomicrobia, with the two phyla Firmicutes and Bacteroidetes being the most represented. Within each phylum, some well-known genera are prominent: *Lactobacillus, Eubacterium, Ruminococcus, Roseburia,* or *Faecalibacterium* within Firmicutes, *Bacteroides* and *Prevotella* within Bacteroidetes, *Bifidobacterium* within Actinobacteria, *Escherichia* and *Desulfovibrio* within Proteobacteria and *Akkermansia* within Verrucomicrobia phylum.

1.2 Observational Studies in Humans Identified the Diet as a Strong Modulator of the Gut Microbiome

Many factors are known to influence the composition and/or activity of the gut microbiome from the first colonization after birth (birthing process, infant feeding method. . .) and throughout the entire life (environment, medication, diet, physical activity, stress. . .) (Cresci and Bawden 2015). Of note, the diet emerges as the most relevant factor in influencing the gut microbiome which composition varies among different populations, cultures, and dietary habits. Evidences about the role of diet and eating habits on the microbiome came with the comparison of fecal microbiota of humans and other mammalian species living in zoos and the wild (Ley et al. 2008). Interestingly, the diet influences bacterial diversity, which gradually increases from carnivory to omnivory and to herbivory. The gut microbiota of humans living a modern lifestyle is typical of omnivorous primates. Another study even suggested that long-term diets can be linked to different gut microbial enterotypes partitioning, dominated by either *Bacteroides* (for diet enriched in animal fat and protein) or *Prevotella* (for diet rich in carbohydrates) (Wu et al. 2011). Besides eating habits, short-term consumption of specific diets entirely composed of animal products also alters the microbiome composition by increasing the abundance of *Alistipes, Bilophila,* and *Bacteroides* and decreasing the levels of some genera/species metabolizing dietary plant polysaccharides (*Roseburia, Eubacterium rectale, Ruminococcus bromii*) (David et al. 2014). Then, the microbiome from US residents with a typical diet rich in protein appears less diverse than microbiome from Malawians and Amerindians consuming diets dominated by corn and cassava (Yatsunenko et al. 2012).

In addition to its impact on the gut microbiota composition, diet also influences the production of gut microbial metabolites and thus the microbiome function (for review see Delzenne et al. (2020)). For instance, the metabolome from vegan individuals contains a higher level of metabolites produced by the gut microbiota and differs from the omnivore's metabolome. This suggests that microbiome can rapidly adapt its structure and function in response to diet. The most well-described bioactive metabolites, produced by the gut microbiota, are the short-chain fatty acids (SCFA, including acetate, propionate and butyrate) generated by microbial fermentation of dietary polysaccharides and having several beneficial properties (energy source for colonocytes, modulation of intestinal inflammation, regulation of metabolism. . .) (Samuel et al. 2008; Donohoe et al. 2011). Unfortunately, some other bioactive compounds resulting from microbiome–diet interactions can have deleterious effects for host physiology, whereas the effects of a large proportion of gut-derived metabolites are still unknown (for review (Rodriguez et al. 2021)).

1.3 Nutritional and Metabolic Disorders Are Associated with Microbiome Changes

Several data support that changes in the composition of the human gut microbiota affect host metabolism and are linked to a variety of diseases (Nicholson et al. 2012). Two important studies highlighted a link between gut bacterial richness and metabolic alterations resulting from nutritional disorders observed during obesity (Cotillard et al. 2013; Le Chatelier et al. 2013). *Cotillard* et al. demonstrated that a diet-induced weight loss (high-protein and high-fibers during 6 weeks and 20% increase in total energy for additional 6 weeks) improved low gene richness and clinical phenotype in obese or overweight people (Cotillard et al. 2013). The fecal gut microbiota from obese individuals was also characterized by a decreased proportion of *Bacteroidetes* versus *Firmicutes* phylum compared to lean people, and this proportion increases upon weight loss (Ley et al. 2006). However, differences in the major phyla proportion is not observed in other cohorts of obese individuals (Duncan et al. 2008). Actually, it seems complicated to draw conclusion based on gut microbiota composition between lean versus overweight/ obese since the dietary habits can obviously be very different between participants. In addition, many other potential cofounding factors (medication, physical activity. . .) can interfere with the interpretation of the data related to the microbiome.

Besides an impact on the bacterial richness or on the major phyla, a regulation in the abundance of certain gut microbial genera or species can be observed in pathologies associated with metabolic disorders. For instance, the comparison of fecal microbiota between obese and lean children/adolescents showed a decreased abundance for some butyrate-producing bacteria belonging to the genera *Ruminococcus*, *Eubacterium,* or *Roseburia* (Zhu et al. 2013). *Bacteroides* abundance was significantly increased and *Prevotella* abundance was decreased in non-alcoholic steatohepatitis patients (NASH) versus patients without NASH, whereas the higher abundance of *Ruminococcus* is found in patients with significant fibrosis (Boursier et al. 2016). Increased level of *Escherichia coli* was also observed in obese patients with NASH versus to whom without NASH (Zhu et al. 2013). Lanthier et al. recently highlighted a reduced abundance of *Clostridium sensu stricto* in obese individuals with a severe fibrosis, compared to patients with a light/ moderate fibrosis, and the abundance of this genus negatively correlated with elasticity measurement (Lanthier et al. 2021). Another example is the decreased abundance of several butyrate-producing bacteria and an enrichment of opportunistic pathogens in T2D subjects (for review (Delzenne et al. 2015)).

Interestingly, a lower intake of carbohydrates decreased concentrations of butyrate and butyrate-producing bacteria (*Roseburia spp.* and *Eubacterium rectale* subgroup of cluster XIV) in feces of obese subjects (Duncan et al. 2007). A higher *A. muciniphila* abundance is associated with a healthier metabolic status in overweight or obese humans (Dao et al. 2016).

Taken together, these data demonstrate that it is difficult to elaborate a precise and recurrent bacterial signature for pathologies resulting from nutritional disorders, since in addition to the difference in dietary habits, many other environmental

confounders may compromise the interpretation of the data. However, it is clear that a gut dysbiosis (corresponding to an alteration of both microbiome composition and function) includes an important reduction of bacteria having the abilities to ferment carbohydrates and to produce short-chain fatty acids.

In order to explain the progression of metabolic disorders following the gut dysbiosis associated with nutritional disorders, preclinical studies have demonstrated that mice fed a high-fat diet had higher plasma lipopolysaccharides (LPS) level associated with gut microbiota changes, higher inflammation, fatty liver, and insulin resistance (Cani et al. 2007). Actually, LPS are important outer membrane components of gram-negative bacteria and their increase in systemic circulation creates a low tone inflammation called "metabolic endotoxemia," this process being linked to an alteration of gut microbiota and thereby, a loss of intestinal barrier function. Thus, the gut microbiome changes and the loss of intestinal barrier integrity could be the starting point to development of metabolic alterations in peripheral organs in response to diet (for review Rodriguez and Delzenne (2021)).

2 Targeting the Microbiome for Improving Metabolic Health in Humans

In line with the previous paragraph, one strategy to envision the improvement of weight control and metabolic alterations resulting from inadequate diet is the manipulation of the gut microbiome with specific dietary advices. In this context, the use of prebiotics, defined as substrates that are selectively utilized by host microorganisms conferring a health benefit (Gibson et al. 2017) represents a major interest. The number of preclinical studies using this approach is very important and the topic has been recently reviewed (Rodriguez and Delzenne 2021). We propose in this section to focus especially on human intervention studies targeting the microbiome to improve metabolic health. The majority of studies evaluating the impact of prebiotics on metabolic health concern supplementation with high-fermentable dietary fibers (inulin-type fructans, resistant starch, arabinoxylans, pectins, or β-glucans) but also with complex food components as polyphenols.

2.1 Inulin-Type Fructans

Inulin-type fructans (ITF) are certainly the most studied prebiotics. They are composed by repetitive fructosyl units linked by β(2,1) bonds and fermented by intestinal bacteria. Their intake has been suggested to alleviate several features of metabolic alterations in preclinical models (including gut permeability, systemic inflammation, or peripheral lipids accumulation) but to date, few studies evaluated their benefit in human health on both the gut microbiota composition and the metabolism (or metabolic disruptions).

In healthy humans, 10 g/day of very-long-chain inulin extracted from globe artichoke (Cynara scolymus), 20 g/day of inulin extracted from chicory root or

16 g/day of ITF (50:50 inulin to fructooligosaccharide FOS mix) increased the bifidobacteria after 2–3 weeks of supplementation (Costabile et al. 2010; Healey et al. 2018; Baxter et al. 2019). In healthy adults, ITF did not systematically increase the SCFA production. No change in the SCFA level was observed upon treatment with inulin from globe artichoke or ITF. Inulin from chicory increased the level of total SCFA but did not significantly modify the individual SCFA (butyrate, propionate, acetate) profile (Costabile et al. 2010, Healey et al. 2018, Baxter et al. 2019). Interestingly, the consumption of ITF-rich vegetables during 3 weeks (with an estimated intake of 15 g/day) also increased the proportion of *Bifidobacterium* genus in healthy adults (Hiel et al. 2019). In addition, the volunteers consuming ITF-rich vegetables showed improvements in food behavior such as a greater satiety and reduced desire to eat sweet or salty.

In children with overweight or obesity, 16 weeks of oligofructose-enriched inulin (8 g/day) decreased bacterial richness, associated with an increase of *Bifidobacterium* and *Collinsella* genera and decreased *Ruminococcus* (Nicolucci et al. 2017). This prebiotic-based intervention improved body fat, the level of interleukine-6, and the triglycerides content in children. Twelve weeks of supplementation with the same prebiotic administer in similar amount increased *Bifidobacterium* and the C-peptide, and was accompanied by a tendency to improve the gut barrier permeability in children with type 1 diabetes (Ho et al. 2019).

In obese adult women, 3 weeks of ITF supplementation increased the *Bifidobacterium* and *Faecalibacterium,* both genera were inversely correlated with the serum LPS, a marker of metabolic endotoxemia (Dewulf et al. 2013). qPCR analysis revealed that ITF especially increased the species *B. longum, B. adolescentis,* and *B. pseudocatenulatum,* and decreased the fecal SCFA, acetate and propionate (Salazar et al. 2015). In a larger cohort, 12 weeks of ITF supplementation (6 g oligofructose + 2 g inulin from chicory root) in adults with overweight/obesity also stimulated the growth of *Bifidobacterium,* whereas food-related behavior was improved with a lower hunger, desire to eat, and prospective food consumption (Reimer et al. 2017). A multicenter placebo-controlled trial performed in obese individuals also confirmed the increased proportion of *Bifidobacterium* genus by ITF associated with ITF-rich vegetables after 3 months (Hiel et al. 2020). This was accompanied by an increase of *Catenibacterium* genus and a decreased proportion of *Desulfovibrio* and *Roseburia* genera. Compared to placebo, the prebiotic induced greater weight loss and additionally decreased diastolic blood pressure, AST, and insulinemia. However, this study identified medication as an important factor to consider during prebiotic-based intervention since metformin use compromised most of the gut microbiota changes and metabolic improvements linked to prebiotic intervention. In a subcohort, ITF did not alter fecal SCFA content but reduced fecal calprotectin, a marker of gut inflammation (Neyrinck et al. 2021). All this data support the increase of *Bifidobacterium* genus as a specific signature of ITF intake. However, the amplitude of *Bifidobacterium* changes, as well as the impact on the host metabolism can vary between the studies and between the individuals involved in a same protocol.

2.2 Galacto-Oligosaccharides

Prebiotic galacto-oligosaccharides, GOS, are polymers of galactose with a terminal glucose monomer. In elderly people, administration of β-GOS mixture (5.5 g/day) enhanced the growth of *Bacteroides* and *Bifidobacterium* and resulted in a higher production of anti-inflammatory cytokine IL10, as well as a lower synthesis of proinflammatory cytokine IL1β, compared to placebo group (Vulevic et al. 2015). The same dose administered for 12 weeks in type 2 diabetes (T2D) individuals had no significant effects on both clinical outcomes or bacterial abundances compared to placebo (maybe due to confounding factors such as medication or an important heterogeneity) (Pedersen et al. 2016). A higher dose of GOS (15 g/day) in overweight or obese people led to an increase of *Bifidobacterium*, without any improvement of metabolic markers (Canfora et al. 2017). This suggests that changing the microbial composition in favor of bifidobacteria growth is not automatically associated with beneficial effects on human metabolism.

2.3 β-Glucans

In patients with high risk of metabolic syndrome, 4 weeks of supplementation with barley β-glucans lowered the plasma total cholesterol (Velikonja et al. 2019). Barley β-glucans also decreased the microbial diversity and increased the production of propionic acid. The prebiotic properties of a novel insoluble fiber chitin-glucan CG, composed by branched β-1,3/1,6 glucan that is linked to chitin via a β-1,4 linkage, were also investigated in healthy humans (Rodriguez et al. 2020b). After 3 weeks of supplementation, CG decreased the relative abundance of *Dorea* and increased the butyrate-producing bacteria belonging to *Roseburia* and *Eubacterium* genera.

2.4 Arabinoxylans

Arabinoxylans (AX) are the most abundant non-digestible carbohydrates present in wheat. An intake of 15 g/day of AX during 6 weeks reduced the gut microbiota diversity in overweight individuals and stimulated the production of total SCFA (Salden et al. 2018). Unfortunately, no changes in metabolic markers (cholesterolemia, triglyceridemia, glycemia, or insulinemia) were observed. Similar results (i.e., bifidogenic effects without metabolic improvements) were obtained after 4 weeks of arabinoxylan oligosaccharides (AXOS) supplementation in overweight people (Kjolbaek et al. 2020).

2.5 Resistant Starch

Resistant starch (RS) is a type of dietary fiber that can be divided into many sub-types (RS1 physically inaccessible, RS2 starch conformation), RS3 retrograded,

RS4 chemically modified or RS5 starch lipid complex), also considered as prebiotics (Gill et al. 2021). In healthy adults, 8 days of RS2-enriched wheat (14-19 g/day) intake altered the overall composition of gut microbiota assessed by β-diversity indices and reduced the α-diversity, a marker of bacterial diversity (Hughes et al. 2021). Compared to baseline, *Ruminococcus, Gemmiger, Faecalibacterium, Roseburia,* and *Bifidobacterium* increased after RS2-enriched wheat supplementation. Interestingly, after 1 week of intervention, some metabolic processes were improved following a challenge with a breakfast containing RS2-enriched wheat (postprandial glucose and insulin response) (Zhang et al. 2019). A higher dose during 4 weeks (40 g/day high amylose RS2) reduced visceral subcutaneous and intra-abdominal fat and promoted early-phase insulin, GLP-1, and acetate production. In contrast to the previous study, RS2 intake did not alter α-diversity. However, it increased the genus Ruminococcaceae_UCG-005 and decreased 15 other bacterial genera. In normotensive and overweight or obese adults, plasma concentration of trimethylamine-N-oxide (TMAO), a biomarker of cardiovascular disease risk and dependent of intestinal microbiota, was higher after a high-RS versus low-RS diet in the context of low carbohydrates intake (Bergeron et al. 2016). Administration of RS3 during a phase of weight maintenance following weight loss improved fasted plasma glucose compared to subjects who did not receive RS (Johnstone et al. 2020). The addition of RS during weight maintenance caused distinct changes by targeting bacterial groups mainly belonging to the genera *Roseburia, Ruminococcus,* and *Faecalibacterium.* It is important to take into consideration that the structure of this dietary fiber is crucial for its impact on the gut microbiota in humans since chemically modified RS with small structural differences induce different specific effect such as the stimulation of different SCFA production (propionate versus butyrate) (Deehan et al. 2020).

To conclude, few human studies investigated the impact of dietary fibers on both the gut microbiota and the metabolic alterations observed in several physiological or pathological conditions. The main issue reported in these first studies is the lack of benefits obtained in humans, particularly for metabolic health, when compared with beneficial effects of dietary fibers supplementation in preclinical models. Some studies explained these disappointing data by an important inter-individual response to dietary intervention targeting the gut microbiota within the studies, leading to the difficult interpretation of results.

3 Can the Gut Microbiome Predict the Efficacy of Dieting in Humans?

Identification of predictive traits for the anticipation of diet-based effects on weight loss is a matter of study in which the gut microbiome emerges as an important factor to take into consideration (see Fig. 1).

The rationale that individualized response to dietary intervention can be dependent on the gut microbiota came from the observation that non-digestible carbohydrates can produce marked changes in the gut microbiota, these changes

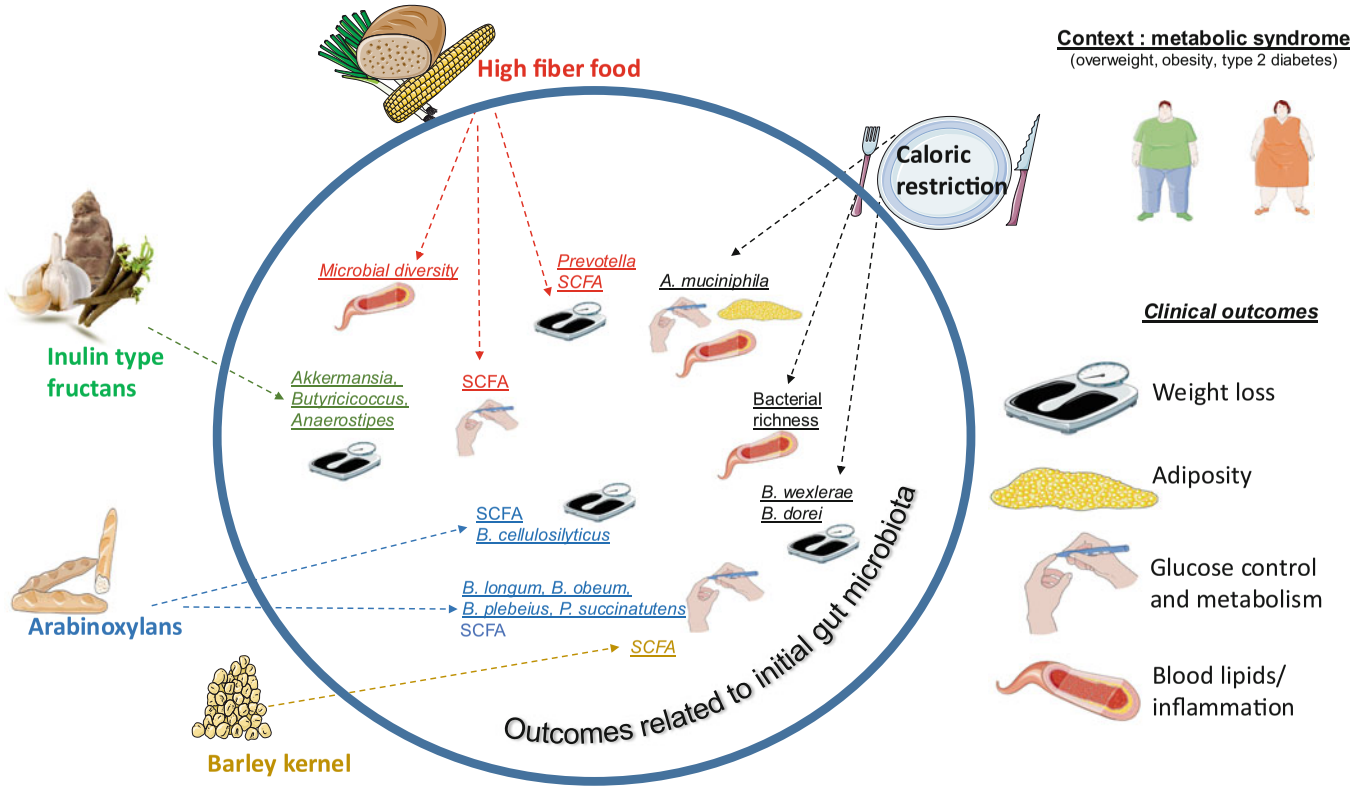

Fig. 1 The gut microbiota is an important factor predicting the metabolic improvements in obese individuals. The figure highlights the gut microbiota characteristics at baseline (richness, presence of specific bacteria, enterotypes, short-chain fatty acids (SCFA) profiling linked to a variable response toward dietary interventions. Metabolic improvements include body weight control, fat mass expansion, glucose homeostasis and blood lipids or inflammation. The type of nutritional intervention is linked to the metabolic outcomes and related characteristics of the gut microbiota

being dependent on its initial composition (Walker et al. 2011). For instance, supplementation with RS in overweight individuals showed that a large difference in the proportion of fecal RS can be found between participants suggesting that the initial microbiota composition can lead to inter-individual differences in microbial fermentation of RS and thus in microbial response. Consistently, a variable response of human microbiome was also observed in another study with RS supplementation, leading to the heterogeneous responses in butyrate concentrations (Venkataraman et al. 2016).

Then, a linear model applied on three different obese cohorts (from Belgium, Finland, and Britain) based on dietary interventions for metabolic health indicated that the baseline abundances of several species (mainly Firmicutes members) can predict the overall responsiveness of the microbiota to the tested interventions (Korpela et al. 2014). In addition to these findings, a 6-month weight-reduction program with collection of dietary, physical activity, body weight, obesity-related host genotypes, and fecal samples identified the baseline gut microbiota as the most powerful individual factor for predicting the individual weight loss trajectories (Jie et al. 2021). In this last study, *Blautia wexlerae* and *Bacteroides dorei* were identified as the strongest predictors for weight loss when they were highly abundant at baseline. Obese/overweight individuals and some lean people composed the cohort, this allowed the authors to show that some species were enriched in obese subjects and their decreased abundance was associated with weight loss (*Coprococcus sp., Holdemanella biformis, Solobacterium moorei, Ruminococcus gnavus*, and *Clostridium sp.).* On the other hand, *Coprobacter* sp., *Bacteroides intestinalis, Akkermansia muciniphila, Alistipes obesi*, and *Tannerella* species were significantly enriched in lean individuals, and their increase during dieting was significantly associated with weight loss. In line with these observations, another study confirmed that initial abundances of some intestinal bacteria can drive the successful of dietary interventions (Dao et al. 2016). Higher basal abundance of *Akkermansia muciniphila* was associated with greater improvements of glucose control, blood lipids, or body composition after caloric restriction. In addition, low gene richness may also have predictive potential for the efficacy of nutritional intervention. Indeed, 6 weeks of energy-restricted high-protein diet followed by a 6-week weight-maintenance diet improved clinical phenotypes, but a less efficient improvement on the inflammatory markers was observed in obese or overweight subjects with lower gene richness (Cotillard et al. 2013).

The hypothesis that gut microbiome composition prior to intervention can influence the response to dietary intervention, and may predict weight loss or metabolic improvement, was reinforced with some data relative to glucose control (Zeevi et al. 2015). A high interpersonal variability in post-meal glucose was observed in an 800-person cohort and the use of personal and microbiome features enables accurate glucose response prediction (Zeevi et al. 2015). Moreover, the initial gut microbiota can influence the glycemic response to bread and the prediction of personal glycemic response-inducing bread can be done by using only the microbiome features (Korem et al. 2017). A recent study using multivariate methods to integrate 24 h-food records and fecal shotgun metagenomes in healthy human confirmed that similar foods can

induce different effects on microbiome, suggesting that the interactions between diet and microbiome are personalized (Johnson et al. 2019). In line with this finding, a recent study also highlighted a personalized immune response to a high-fiber supplementation in healthy adults (Wastyk et al. 2021). In this work, the authors identified three different clusters associated with distinct immunological trajectories in high-fibers consumers. The different immune response to high-fiber diet seems to be linked with baseline microbiota diversity, the higher diversity at baseline being observed in the group exhibiting the lower inflammation during the study.

Microbial enterotypes have also been proposed as a tool for predicting weight loss during a nutritional intervention. For instance, subjects with a high *Prevotella/Bacteroides* (P/B) ratio had improved enzymatic capacity for fiber digestion and glucose metabolism after 3 days of barley kernel-based bread, compared to subjects with a low P/B ratio (Kovatcheva-Datchary et al. 2015). This suggests that *Prevotella* plays an important role in the barley kernel-induced improvement in glucose metabolism. Other studies showed a link between *Prevotella* abundance in the human gut microbiota and weight loss when consuming fiber-rich diet in healthy or overweight subjects (Christensen et al. 2019; Hjorth et al. 2019). Interestingly, subjects with high P/B ratio were more susceptible to weight loss on a diet rich in fiber, compared with subjects with low P/B ratio (Hjorth et al. 2019). These observations underline that the P/B ratio could be an important biomarker within personalized nutrition for weight management (Hjorth et al. 2019). Another study confirms that P/B ratio can predict weight change in overweight people after 4 weeks of AXOS supplementation, but suggests that few species from *Bacteroides* genus, owing to AXOS-degrading capacity, would predict body weight changes (Christensen et al. 2020). The authors found association between *B. cellulosilyticus* and metabolic changes in overweight subjects consuming AXOS. The inter-individual response of gut microbiome to AXOS supplementation was also observed in healthy adults (Chung et al. 2020). The authors reported a different gut microbiota response to AXOS in individuals with higher levels of *Prevotella* at baseline compared to whom with lower abundance of this genus. However, no changes in SCFA production or metabolic markers were observed. In addition, AXOS supplementation in overweight people also induced a variable response in terms of propionate production (Nguyen et al. 2020). Propionate response was predictable through baseline composition of gut microbiota and after 6 weeks of intervention, propionate responders and non-responders differed in their microbiome response to AXOS.

In line with the importance of some bacteria as drivers of response to dietary response, Zhao et al. highlighted that a set of SCFA-producing bacteria, promoted by dietary fibers, was crucial for improving host glycemic control (Zhao et al. 2018). Interestingly, when these SCFA producers were present in greater abundance, T2D participants had better improvement in hemoglobin A1c levels. Another study demonstrated a different metabolic response to inulin supplementation in mice inoculated with stool samples from different obese donors with different gut microbiota characteristics (bacterial richness, level of *Bifidobacterium sp.*) (Rodriguez et al. 2020a). Interestingly, the gut microbiota from obese individuals

who exhibited a beneficial response to inulin in terms of BMI improvement was characterized by greater abundance of *Akkermansia* and *Butyricicoccus* and lower level of *Anaerostipes.*

In addition, it is also unanswered if long-term consequences from dieting in terms of weight loss maintenance would also be influenced by the gut microbiota. A recent study compared the impact of healthy dietary guidelines, Mediterranean, and Mediterranean/high polyphenols diet on the weight control of abdominally obese or dyslipidemic participants, their gut microbiome composition but also the regain of the weight loss after the intervention (Rinott et al. 2021). In this study, the Mediterranean/high polyphenols diet was the only dietary strategy inducing a significant change in microbiome composition during the 6 months of weight loss phase. Interestingly, by administering autologous fecal material transfer collected at the end of 6-months intervention and for additional 8 months, the authors observed that participants from Mediterranean/high polyphenol group had an attenuated weight regain, waist circumference, and a reduced insulin rebound compared to a placebo administration. This suggests that the maintenance of an "optimal" microbiome composition obtained by dietary intervention can help to also preserve the weight loss and metabolic improvements obtained after the nutritional program.

4 Conclusion and Perspectives

In conclusion, few human studies investigated the impact of dietary fibers on both the gut microbiota and the metabolic alterations observed in several physiological or pathological conditions. The main issue reported in these first studies is the lack of benefits obtained in humans, when compared with beneficial effects of dietary fibers supplementation in preclinical models. This can probably reflect an important inter-individual variation in response to the dietary fibers, due to the baseline gut microbiota composition, but also the presence of several confounding factors to take into account in the interpretation of the clinical studies, these factors being strictly controlled in preclinical experiments (similar diet, activity, housing. . .). In summary, it seems that the gut microbiota contains critical information for identifying the optimal health outcome toward dietary intervention and the gut microbiota characteristics may thus be used in the future for personalized food-related recommendations. Predicting how the gut microbiome will respond to a dietary intervention and identifying all the confounders susceptible to influence the metabolic response to intervention is the future challenge in personalized nutrition.

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From Obesity to Diabetes: The Role of the Adipose Organ

Georgia Colleluori, Jessica Perugini, Antonio Giordano, and Saverio Cinti

Contents

Abstract

Obesity is a complex, multifactorial, and relapsing disease whose prevalence has tripled during the last decades and whose incidence is expected to further increase. For these reasons, obesity is considered as a real pandemic, deeply burdening the global health-care systems. From a pathophysiological standpoint obesity is the result of a chronic-positive energy balance which in turn leads to an excessive accumulation of lipids, not only within the adipose organ, but also in different cytotypes, a phenomenon leading to lipotoxicity that deeply compromises several cellular and organs functions. Obesity is therefore associated with over 200 medical complications, including insulin resistance and type 2 diabetes mellitus (T2DM) and represents the fifth leading cause of death worldwide. In this review, we describe the main pathophysiological

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mechanisms linking obesity-induced adipose organ dysfunction to insulin resistance and T2DM.

Keywords

Adipocytes dysfunction · Adipose tissue · Insulin resistance · Obesity

Obesity, defined as a body mass index (BMI) $\geq$30 kg/m^2, is a complex, multifactorial, and relapsing disease (Bluher 2019; Ward et al. 2019). Obesity and overweight prevalence is estimated to reach nearly 40% of the adulthood population in numerous countries (e.g., USA, Canada, Australia, UK, and Saudi Arabia) (NCD-RisC 2021) and their incidence is expected to further increase during these coming decades (Bluher 2019; Ward et al. 2019; EASO 2020). Children with obesity are in fact steeply rising (WHO 2021a) and tend to maintain the disease throughout adulthood (Geserick et al. 2018). For these reasons, obesity is considered as a real pandemic, deeply burdening the global health-care systems (WHO 2021b; Bray et al. 2017).

From a pathophysiological standpoint obesity is the result of a chronic-positive energy balance which in turn leads to an excessive accumulation of the excess of lipids, not only within the adipose organ, but also in other cytotypes, a phenomenon resulting in lipotoxicity and deeply compromising several organs functions (Bray et al. 2017; Batsis and Villareal 2018). Obesity-induced organ dysfunction impairs whole-body energy homeostatic abilities, hence triggering a vicious cycle at the basis of the relapsing nature of the disease. It therefore does not surprise that obesity is a chronic illness associated with over 200 medical complications, including insulin resistance, type 2 diabetes mellitus (T2DM), hypertension, cardiovascular events, metabolic syndrome, and certain types of cancers (e.g., breast and colon cancer), and that it represents the fifth leading cause of death worldwide (Bluher 2019; Bray et al. 2017).

In this review, we describe the main pathophysiological mechanisms linking obesity, in particular adipose organ dysfunction, to insulin resistance.

1 White and Brown Adipose Tissues

White adipocytes are the main cell component of the white adipose tissue (WAT) whose core function is to store energy to supply other tissues and to release hormones modulating the whole-body energy balance (Cinti 2012, 2019). On the other side, brown adipocytes are organized to form the brown adipose tissue (BAT), whose main role is to burn lipids for thermogenetic purposes (Cinti 2012, 2018a, 2019; Frontini et al. 2007; Cannon and Nedergaard 2004). WAT and BAT are organized to form the adipose organ which occupies body's visceral and subcutaneous regions (Cinti 2012, 2019). The adipose organ finely cooperates with digestive organs and central nervous system nuclei regulating eating behavior with the

common objective to guarantee the nutritional and energetic homeostasis (recently referred to as the nutritional system) (Colleluori et al. 2021).

1.1 White Adipose Tissue

White adipocytes are spherical cells of about 70–100 μm in diameter, mainly composed of a single lipid droplet composed of triglycerides occupying ~90% of their volume (Fig. 1a). The sphere is the geometrical form including the largest volume in minimal space, which makes it ideal for adipocyte functional purposes: provide energetic fuel for the organism during the intervals between meals. Humans' history has been characterized by famine and starvation, reason for which such cells have played a crucial role in guarantying survival during the long fasting intervals. Interestingly the fuel is composed by the fatty acids forming triacylglycerol: molecule containing the highest density of energy to serve organs with a high demand such as the heart. On the other side, during fasting, white adipocytes also produce a hormone called asprosin (Romere et al. 2016) which is in turn able to stimulate glucose secretion by the liver to maintain glycemic homeostasis.

White adipocyte anatomy is very simple being composed only of a thin cytoplasmic rim surrounding the lipid droplet and of a squeezed, crescent-shaped nucleus, both occupying the 10% of the cell volume. Few elongated and small mitochondria with random oriented short cristae are the most visible organelle found in adipocytes' cytoplasm. Rough endoplasmic reticulum (RER) and Golgi complex are rarely observed: RER is in fact usually represented by single short strands, while Golgi complex is small; considering cell's size, a random section often does not allow to visualize them during electron microscope analyses. Several small vacuoles of various sizes (30–150 nm) are often found, mainly in the sub-plasmalemmal area. Recently a microvesicular compartment (30–40 nm) has been identified as the site containing the secretory microRNAs (Isaac et al. 2021).

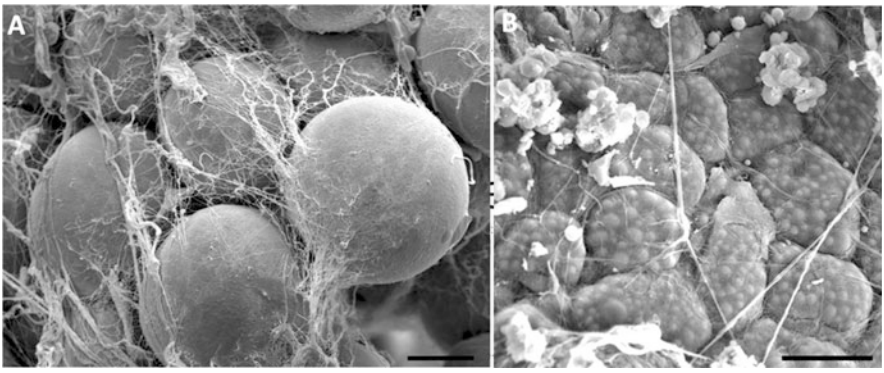

Fig. 1 Scanning Electron Microscopy. (**a**) Mouse white adipocytes. (**b**) Mouse brown adipocytes. Bars: 20 μm

Basal membrane lies close to the outer side of each adipocyte's plasmalemmal. The main component of this structure is collagen IV, and its morphology recalls a felt-like membrane of about 150–200 nm (human adipocytes). On the external side of this membrane a network of collagen fibrils mainly of collagen type III is always present. This network is well visible by scanning electron microscopy (Giordano et al. 2013).

Importantly, white adipocytes are endocrine cells. Together with several hormones and cytokines (~600) that play important roles in glucose and lipid metabolism, coagulation, growth, and immunity, they produce a couple of hormones able to influence the most important behavior of humans regarding food search and intake: leptin and asprosin.

Leptin was discovered in 1994 and its production is related to the amount of fat (Zhang et al. 1994). People with low leptin plasma levels are pushed to find food because this hormone acts on limbic system affecting eating behavior (De Matteis and Cinti 1998). Until few years ago it was generally thought that low leptinemia was sufficient to induce food intake. However, the study of the gene responsible for a severe form of congenital lipodystrophy revealed that adipocytes produce asprosin, an adipokine required to induce food intake in condition of low leptin levels (Romere et al. 2016). Therefore, white adipocytes produce very important hormones that, acting on brain, induce critical behaviors ensuring animal's survival: leptin to induce food search and asprosin to induce food intake.

White adipocytes are the main cell type of WAT, but several other parenchymal cytotypes are also present together with cells of the innate immunity and can be studied separating the stromo-vascular fraction (SVF) from mature adipocytes. Since adipocytes are post-mitotic cells, WAT hyperplasia can occur through precursors proliferation and then differentiation into mature adipocytes. Numerous efforts have been put into the study of early adipocyte precursors. In 2008, Rodeheffer and colleagues identified an early adipocyte progenitor subpopulation (Lin-:CD29+:CD34+:Sca-1+:CD24+, representing the 0.08% of the SVF) able to reconstitute normal WAT and rescue the diabetic phenotype when injected in A-Zip lipodystrophic mice (Rodeheffer et al. 2008). Additional evidence revealed the presence of a stem cell pool of adipocytes precursors, highly mesenchymal in phenotype, proximal to the blood wall within the adipose depot (Muller et al. 2016; Zimmerlin et al. 2013). The presence of adipocytes early precursors and preadipocytes in WAT is necessary considering that the average lifespan of human adipose cells is about 10 years (Spalding et al. 2008). Preadipocytes morphology is highly characteristic: small poorly differentiated cells with a high nucleus/cytoplasmic ratio, closely associated with capillary wall and surrounded by an external lamina similar to that above described. Preadipocytes cytoplasm is poor in organelles: it is mainly represented by few mitochondria with the general characteristics like the above described for mature cells, and by many ribosomes and polyribosomes, occupying most of the cytoplasmic area. Small lipid droplets are usually present, but they tend to coalescence, thus in the intermediate forms of maturation, preadipocytes are usually composed by a single droplet occupying most of the cytoplasm. Their close relationship with capillary wall is in line with the

hypothesis that adipose stem cells arise from the vascular components (Tran et al. 2012). Our and others' data support the idea that the adipose stem cells are the endothelial cell of adipose tissue capillaries. Morphology data showed intermediate forms between endothelial cells and pericytes that are considered an obligatory step toward preadipocytes by most authors (Tran et al. 2012). Furthermore, lineage tracing technique (allowing recognition of dynamic evolution and developments in mammalian tissues) supported this origin both in white and brown adipose tissues (Tran et al. 2012).

Together with the great importance of vasculature, parenchymal noradrenergic nerve fibers also play a crucial role in both BAT and WAT plasticity phenomena (see below).

Several types of immune cells are usually present in WAT: mast cells and histiocytes (macrophages) are the most abundant. Mast cells are usually present in close proximity to vascular wall in line with their main function of vascular regulators. Their morphology is unique and allows an easy recognition by transmission electron microscopy. Large granules (0.5–1.0 mm) with typical electron-density and structural membranous appearance occupy most of their cytoplasm. Alteration in mast cell density and granule ultrastructural appearance may be related to their role in regulating the histamine-dependent vasodilation. Interestingly, histamine was demonstrated to promote WAT browning (Zhao et al. 2019) and regulate BAT thermogenesis (Karlstedt et al. 2003). Vasodilation and thermogenesis coupling is in fact required to distribute the produced heat to other organs.

WAT macrophages are abundant and are found in proximity of adipocytes. They are small with a high nucleus/cytoplasmic ratio. Their nucleus is always indented and their surface is highly irregular due to several cytoplasmic elongation of variable length. Several primary and secondary lysosomes occupy a relevant proportion of the cytoplasmic area, especially in proximity of the Golgi complex that is usually quite evident.

1.2 Brown Adipose Tissue

Brown adipocytes are different from white adipocytes (Fig. 1b). They are smaller (about 1/3 of white adipocytes size) and polygonal. Their nucleus is regularly roundish and often in central position. Mitochondria are the most represented organelles. They are numerous, large, and rich in laminar cristae extending from one side to the other, and with a density that depends on their functional activity. Several small lipid droplets formed by triglycerides are also present among mitochondria. Their number and size depend on cell functional activity: smaller and numerous in active cells, large and few in inactive or poorly active adipocytes. A basal membrane, similarly, to the above described for white adipocytes, is also present in brown adipocytes. This cell uses triglycerides to produce heat and the main stimulus for their thermogenic activity is cold exposure. Cold activates the sympathetic nervous system that, through noradrenergic fibers directly contacting brown adipocytes, activates specific β receptors (β3) on their plasmalemma. The

stimulus is transferred to the surrounding cells through gap junctions that increase cells size in stimulated BAT (Barbatelli et al. 1994). The activation signaling involves G proteins, cAMP and PKA activation with three main consequences: (i) activation of mitochondrial uncoupling protein (UCP1) through fatty acids deriving from increased lipolysis; (ii) neosynthesis of UCP1; (iii) mitochondriogenesis. Such phenomena result in an increased thermogenesis: energy dissipation due to the uncouple of the electron transport chain and oxidative phosphorylation made by UCP1. Importantly, BAT activation results in the upregulation of the gasotransmitter nitric oxide (NO), which is able to induce vasodilation, necessary to transport the produced heat to other organs (Roberts et al. 2015; Giordano et al. 2002). The multilocularity of lipids is necessary to increase lipid droplets surface being it the site where lipolysis occurs. This strategy allows the entry of a large amount of fatty acids in the numerous mitochondria and a very high oxidative process that does not converge into the generation of ATP, but to heat production (for details see also chapter by Barbara Cannon in this book).

Together with the high parenchymal density of noradrenergic fibers, BAT has also a high density of vascular supply. A vascular density of BAT was estimated to be around six times higher than the one in WAT (Nechad 1986). The BAT high vascular density is justified by two main needs: i. supplies a high amount of oxygen for the metabolic purposes and ii. a rapid removal of the heat to avoid cellular damages.

BAT has complementary parenchymal cell population like those described for WAT, but with more mast cells for vascular control and with less immune cells.

2 The Adipose Organ

WAT and BAT are organized to form a true organ (Fig. 2a). The definition of organ as "*a dissectible structure composed by at least two tissues with cooperative functions*" is easily applicable for the adipose organ. Several experiments of anatomical dissection demonstrated its dissectability in several different physiologic conditions, including cold and warm exposure, fasting, obesity, and pregnancy (Cinti 2018b). WAT and BAT are easily recognizable macroscopically: WAT is predominant in both the subcutaneous and visceral compartments, while BAT is well represented (mainly in cold conditions) in the interscapular, subscapular, cervical, and axillary areas of subcutis and in the periaortic and perirenal regions in the visceral compartment. The continuity between the subcutaneous and visceral compartment are visible in Fig. 2: in the upper part of the body, visceral mediastinal fat follows the subclavian arteries departing from the aortic arch. In the bottom part of the body, visceral pelvic fat is in direct continuity with the subcutaneous part (gluteal) at the level of the ilio-ischiatic junction, corresponding to the greater ischiatic incisura in humans.

Even in humans, the adipose organ is organized into distinctive adipose compartments (visceral adipose tissues – VAT – and subcutaneous adipose tissues – SAT –) and depots (e.g., omental, perirenal, epicardial, etc.) that can be

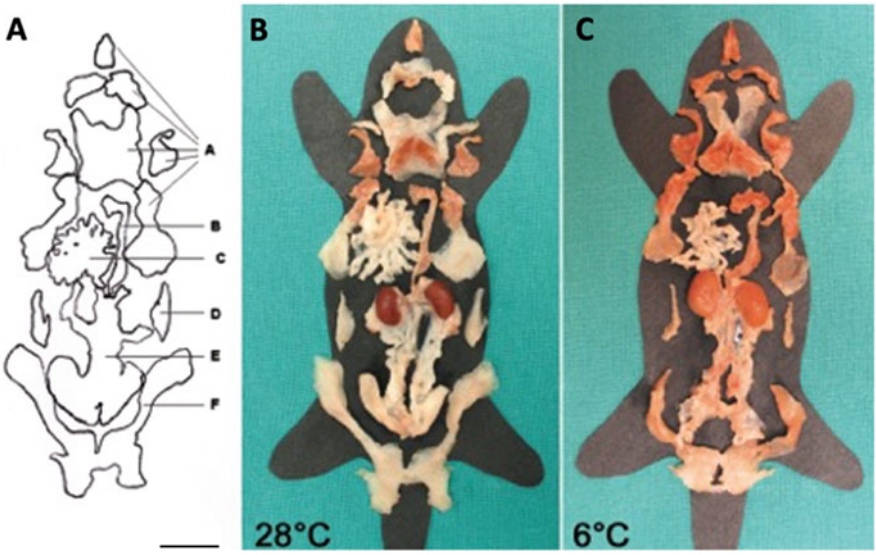

Fig. 2 Mouse Adipose Organ of adult female mice maintained at different temperatures for 10 days. (**a**) A: anterior subcutaneous fat depot, B: mediastinal-periaortic visceral fat depot, C: mesenteric visceral fat depot, D: retroperitoneal visceral fat depot, E: abdomino-pelvic fat depot, F: posterior subcutaneous fat depot. (**b**) Mouse adipose organ anatomy at 28°C. (**c**) Mouse adipose organ anatomy at 6°C. From Murano et al. Adipocytes 1: 121–130, 2005 with permission. Bar: 12 mm

macroscopically identified. Visceral-subcutaneous junctions are located in sites corresponding to those described above for the murine adipose organ, while the BAT component is mainly located in the supraclavicular area in the subcutaneous part and in the perirenal region in the visceral component.

3 Adipose Organ Plasticity

Based on the different physiologic conditions, adipose organ composition varies: the cellular phenomena underlying such variation define the cooperative requirement requested by the organ definition. During cold acclimatization, the adipose organ undergoes the phenomenon of browning (Fig. 2c). Such phenomenon occurs thanks to the differentiation of progenitors into mature brown adipocytes (Lee et al. 2012; Shan et al. 2013) and, based on our qualitative (Barbatelli et al. 2010; De Matteis et al. 2009; Cousin et al. 1992) and quantitative data (Vitali et al. 2012), to the direct transdifferentiation of white into brown adipocytes. Such phenomenon was also recently confirmed by lineage tracing experiments (Rosenwald et al. 2013). Importantly, during obesity the adipose organ undergoes the reverse process: whitening (Kotzbeck et al. 2018). Additional lineage tracing experiments suggested the occurrence of the transdifferentiation phenomenon during pregnancy and lactation,

moments at which part of the mammary gland adipocytes convert into milk secreting epithelial alveolar cells (Morroni et al. 2004; Giordano et al. 2017), a process supported by explants experiments (De Matteis et al. 2009), but still controversial in the literature based on recent findings (Colleluori et al. 2021).

4 The Adipose Organ in Obesity Disease

In obesity, the chronic caloric excess is stored in the form of lipids in the adipose organ which expands through adipocyte hypertrophy and hyperplasia (Kim et al. 2014). Adipocyte volume in humans is positively related to total fat mass, while adipocyte number is set during adolescence and remains relatively stable throughout life (Muller et al. 2016; Spalding et al. 2008). Consistently, adipocyte hypertrophy, but not hyperplasia is observed in healthy adults after 4 months of weight gain (Salans et al. 1971), while massive weight loss by bariatric surgery results in the reduction of adipocyte size, but not number (Spalding et al. 2008). However, adipocyte hyperplasia has been detected in adulthood, specifically in VAT depots upon sustained positive energy balance, moment at which adipocytes cannot further accommodate the extra calories increasing their size (Kim et al. 2014; Wang et al. 2013). Lipids are in fact first preferentially deposited in SAT, and then, once SAT storage abilities are saturated, they are stored in VAT and ectopic depots (e.g., liver, skeletal muscle, pancreas, heart), a phenomenon representing the *primum movens* of lipotoxicity (Despres and Lemieux 2006). Differently from SAT, VAT and ectopic fat localizations are in fact associated with insulin resistance, T2DM, dyslipidemia, chronic inflammation, and hypertension (Tchkonia et al. 2013; Siervo et al. 2016; Brinkley et al. 2012; Goodpaster et al. 2000), all conditions that, based on clinical observations, increase subjects mortality risk independently of BMI (Cerhan et al. 2014; de Hollander et al. 2012; Santanasto et al. 2017). On the other side, SAT adipocyte hypertrophy is correlated with low adipocyte generation rate which in turn predicts insulin resistance in humans and animal models (Spalding et al. 2008; Kim et al. 2014; Weyer et al. 2000; Arner et al. 2010). Obesity is in fact characterized by preadipocyte dysfunction and reduced ability to perform de novo adipogenesis, a feature contributing to the reduced adipose depot expandability and at the basis of the metabolic anomalies related to the disease (Guo et al. 2007; Sepe et al. 2011). SAT SVF adipogenic potential is reduced among prediabetic and diabetic subjects with obesity compared to obese individuals with normal glucose control, while SAT and VAT adipogenic precursors are lower among the first two groups as compared to the last one (Belligoli et al. 2019). Collectively, the above data support progressive human WAT dysfunction evident not only comparing normo-weight and obese subjects, but also within the obese population comparing those with different glucose control.

Adipocyte plasticity, hence expansion abilities, is in fact of particular relevance: adipocytes become hypertrophic until a *critical size* threshold, specific for each depot, is reached; above such threshold cells cannot further expand, display anomalies in fatty acid flux, signs of stress, and die of pyroptosis (Giordano et al.

2013; Cinti et al. 2005). In this condition, hypertrophic adipocytes are also characterized by alterations in the adipokines and chemokine expression pattern (i.e., lower adiponectin, higher TNF-α, IL-6, MCP1, and resistin), which in turn attract pro-inflammatory immune cells, resulting in inflammation and insulin resistance (Giordano et al. 2013; Lumeng et al. 2011). Accordingly, a particular adipocyte size threshold associated with T2DM and low metabolic response to gastric bypass was identified in patients suffering from obesity (Cotillard et al. 2014). Adipocyte's stress and death is hence accompanied by inflammatory cells infiltration: pro-inflammatory macrophages absorb cell remnants/debris and surround dead adipocytes forming the so-called *crown-like structures* (-CLS- Fig. 3) (Cinti et al. 2005; Murano et al. 2008). Hence, it is not surprising that CLS are more prevalent in obese than normo-weight subjects (Cinti et al. 2005) and that their presence in the adipose tissue is associated with lower insulin sensitivity, higher VAT size, ectopic fat infiltration (liver and muscle), and circulating inflammatory cytokines (e.g., TNF-α) (Le et al. 2011). Importantly, the strong association between visceral obesity and insulin resistance may be in part explained by the lower *critical death size* of visceral adipocytes, which therefore display lower plasticity and tissue remodeling abilities compared to the ones belonging to SAT depots (Giordano et al. 2013; Murano et al. 2008). In addition, as VAT discharges the free fatty acids excess directly into the portal circulation, liver fat accumulation is observed in condition of visceral obesity and often results in the *non-alcoholic fatty liver steatohepatitis* (NASH) also associated with liver dysfunction and insulin resistance. Circulating free fatty acids then reach the skeletal muscle leading to tissue inflammation and dysfunction, strongly contributing to systemic insulin resistance (Wu and Ballantyne 2017). In addition, pancreatic lipids deposition in condition of chronic-positive energy balance also occurs and deeply compromises β-cell function, hence glucose tolerance (Lee et al. 2009). Based on the above phenomena, the beneficial effects of PPARγ agonists (thiazolidinediones), drugs able to reduce circulating free fatty acids increasing adipose expandability, for the treatment of T2DM are not surprising (Rosen and Spiegelman 2006).

Adipocyte's hypertrophy is related to tissue remodeling anomalies such as insufficient angiogenesis, fibrosis, and hypoxia, all phenomena responsible for reduced tissue plasticity and functional alterations (Sun et al. 2013; Cancello et al. 2005; Goossens et al. 2011). Interestingly, adipose tissue fibrosis predicts response to Roux-en-Y Gastric Bypass with the higher fibrosis being associated with poorer weight loss response (Bel Lassen et al. 2017). Clément's group demonstrated that obesity is characterized by an increased WAT prevalence of a subpopulation of collagen producing PDGRFα+ adipose precursors which may act synergistically with the increased TGFβ levels leading to WAT fibrosis, local and systemic metabolic alterations (Marcelin et al. 2017). It is worth noting that SAT and VAT capillary density is lower among patients with obesity compared to normo-weight individuals, independently of their glycemic control, but that capillary basal membrane was thicker among individuals with prediabetes and T2DM, a feature that may explain the microvascular complications associated with such metabolic anomalies (Belligoli et al. 2019). The age-related fat redistribution in favor to visceral depots in

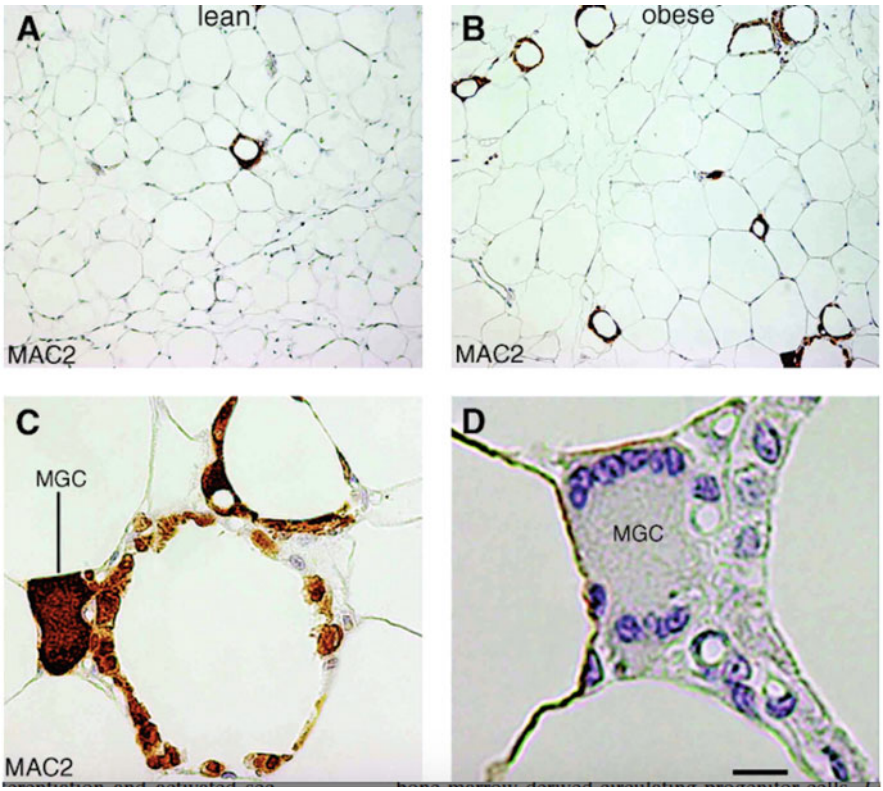

Fig. 3 White adipose tissue (WAT) macrophages form crown-like structures (CLS) around individual adipocytes, which increase in frequency with obesity. Light microscopy of visceral WAT of lean (**a**) and obese db/db (**b**) mouse showing MAC-2 immunoreactive macrophages (brown color) aggregated to form rare (**a**; lean) or numerous (**b**; obese) CLS among unilocular adipocytes. Note that almost all MAC-2 immunoreactive macrophages are organized to form CLS. (**c**) Enlargement of the bottom right corner of (**b**) showing that almost all mononuclear cells in CLS are MAC-2 immunoreactive (i.e., activated macrophages). Note the multinucleate giant cell (MGC), which stains intensely for MAC-2. (**d**) Serial section consecutive to that shown in (**c**) confirming the presence of multiple nuclei (blue) in the MGC. Bar: 100 μm for **a**, **b**, 28 μm for **c**, and 10 μm for **d**. From Cinti et al J Lip Res 2005 Nov;46(11):2347–55 with permission

part attributed to sex hormonal profile modifications (Aguirre et al. 2015; Colleluori et al. 2018a, b; Jeffery et al. 2016), and strongly contributes to the reduced glucose control among elderly (Santanasto et al. 2017). Surprisingly, SAT, but not VAT, senescence revealed by β-Gal staining was demonstrated to be a hallmark of impaired glucose control independently of age which however did not affect response to bariatric surgery among severely obese subjects (Rouault et al. 2021). The lack of link between SAT senescence and chronological aging emphasizes the need of identifying new aging independent factors responsible for WAT senescence.

5 Adipose Organ Dysfunction, Inflammation, and T2DM in Obesity

In 2003, two American groups made a milestone discovery: the obese adipose organ is inflamed, and macrophages are the main inflammatory cells (Weisberg et al. 2003; Xu et al. 2003). Based on their studies, the size of adipocytes and the number of infiltrating macrophages are associated with insulin resistance and fat inflammation. The longstanding clinical observation linking obesity and T2DM was thought to be due to the increased production of cytokines, especially of TNF-α, by obese adipocytes (Bjorntorp and Rosmond 1999). In line with this hypothesis inflammatory cytokines were showed to be responsible for the altered phosphorylation of a serine residue of the insulin receptor substrate 1 (IRS1), hence contributing to the insulin resistance in animals and humans with obesity (Hotamisligil et al. 1996). The work by Anthony Ferrante's and Hong Chen's groups demonstrated instead that the main source of TNF-α and of other cytokines responsible for the insulin resistance are macrophages and not obese adipocytes. However, the cause for the increased number of macrophages in obese WAT was not clear. Our data showed that obese adipocytes hypertrophy leads to cell death and that the need for cleaning residual cellular debris results in macrophage infiltration (Giordano et al. 2013; Cinti et al. 2005; Murano et al. 2008). Importantly, TNF-α is not the only cytokine influencing glucose metabolism. Recently, galectin-3, a lectin produced by macrophages, was also demonstrated to promote adipose tissue inflammation and glucose intolerance probably through impaired neoadipogenesis (Blasetti Fantauzzi et al. 2020). M1 macrophage-secreted exosomal miRNA155 can induce insulin resistance, but M2 macrophage-secreted exosomal miRNA690 improves insulin sensitivity in obese mice (Ying et al. 2019, 2021). Furthermore many other cytokines can induce insulin resistance (Ying et al. 2021; Hotamisligil 2017) and the common signaling pathways involved include both NF-κB and JNK activity (Arkan et al. 2005; Hirosumi et al. 2002). Macrophages can influence adipose innervation (Wolf et al. 2017) and adipocytes converting to brown produce high levels of neurotrophin 3 which contributes to nerve growth (Cui et al. 2021), hence the vascular support can be affected, and vascular supply and fat oxygenation have been showed to play an important role in influencing glucose metabolism. Inflammation determines the onset of insulin resistance, the first step toward T2DM (Hotamisligil 2017). Insulin resistance usually persists several years before T2DM onset, as pancreatic beta cells can compensate such phenomenon through insulin overproduction (DeFronzo et al. 2015). The recent discovery that bariatric surgery can restore glucose metabolism before the occurrence of a significant weight loss opened new avenues to the interpretation of these pathophysiologic events (Cefalu et al. 2016). However, we found that in both obese mice and humans, insulin secretion by pancreatic beta cells is inhibited by the increased number of noradrenergic parenchymal nerve fibers in direct contact with beta cells (Cinti et al. 2021; Giannulis et al. 2014).

6 New Browning Drugs

Obesity management is highly effective in delaying the progression from prediabetes to T2DM and in improving glucose control among diabetic subjects (American Diabetes Association 2021). The first line of therapy consists of lifestyle interventions (i.e., behavioral therapy, diet and exercise) (American Diabetes Association 2021) which result in weight loss and metabolic outcomes improvements (Look et al. 2013; Look 2014). However, given the relapsing nature of obesity, lifestyle interventions not always lead to long-term results (Toplak et al. 2015). For this reason, additional therapeutic strategies are highly necessary to face obesity pandemic. The use of anti-obesity medication, several of which exert a central action modulating appetite and satiety, is adopted to help patients adhering to the dietary recommendations and to sustain the long-term weight loss and metabolic improvements (American Diabetes Association 2021; Toplak et al. 2015; Pasquali et al. 2020). However, the number of effective drugs to face obesity is very scarce (Toplak et al. 2015). As adipose organ browning results in an increase in energy expenditure, it can be exploited to counteract adipocytes hypertrophy (Giordano et al. 2016). Several efforts are hence made by pharmaceutical companies to identify effective browning drugs. Considering that not only BAT, but also WAT (in rodents and humans) expresses the β3 receptors, and in light of the role of β3 receptors in promoting BAT activation, β3 agonists have been synthetized and tested (De Matteis et al. 2002). Although the first developed drugs failed to obtain significant clinical results (Larsen et al. 2002; Redman et al. 2007), mirabegron, from the last generation of β3 agonists, was proved to effectively activate human BAT (Cypess et al. 2015) and to ameliorate several parameters of metabolic syndrome (O'Mara et al. 2020). Interestingly, mirabegron induces G-protein coupled receptor (GPCR) activation (Cypess et al. 2015). Since around 30% of the FDA approved drugs target GPCRs (Hauser et al. 2017), it is possible that some of those drugs have browning properties, which is worth further exploring. Brown adipocytes express about 20 receptors that are coupled to G proteins (Klepac et al. 2016). Adenosine receptors are examples of brown adipocytes' GPCRs, able to activate cAMP signaling independently of β receptors (Hauser et al. 2017). Importantly, it was shown that adenosine signaling exploiting the A2A or A2B receptors is required for the cold induced, BAT-dependent, energy dissipation. Activation of these receptors with small molecule agonists significantly increases whole-body energy expenditure in mice (Gnad et al. 2014, 2020). Recently it has been shown that specific subpopulations of adipocytes generate acetate that locally stimulates GPCR43, a GPCR controlling thermogenic function in brown/beige adipocytes (Sun et al. 2020). Furthermore, a new compound called BIBO3304, with selective antagonism of peripheral Y1R receptor (Y1R), was recently shown to induce a significant reduction in body weight due to enhanced energy expenditure. Administration of this compound increased BAT thermogenesis and induced an extensive WAT browning in mice and humans. Importantly, selective ablation of Y1R from adipocytes protected against diet-induced obesity. Furthermore, inhibition of peripheral Y1R was demonstrated to improve glucose homeostasis mainly affecting Akt activity in BAT (Yan et al.

2021). Importantly, as studies from the last decade have emphasized the existence of different BAT adipocytes' subtypes with specific metabolic functions (Cohen and Kajimura 2021), research efforts should be centered on the formulation of therapeutic strategies selectively targeting brown adipocytes subpopulations able to improve obesity-related complications.

7 Conclusion

Over the last decades, the comprehension of the molecular mechanisms underlying the obesity-induced adipose organ dysfunction has disclosed key mechanisms leading to insulin resistance and T2DM onset and has allowed to generate some targeted-oriented therapies. Obesity-related complications are in fact responsible for the elevated mortality rate among this population, reason for which treatments going beyond the mere weight loss such as the use of GLP-1 agonists are of great clinical relevance (EASO 2020; Pasquali et al. 2020). In the next years, research is expected to provide crucial insights on the characterization of (1) distinct adipocytes subpopulations with specific functions within adipose depots (Sun et al. 2020; Sarvari et al. 2021), (2) the endocrine role of adipose depots, i.e., their crosstalk with brain and peripheral organs.

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Conflict of Interest Authors have nothing to disclose.

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Targeting Epicardial Fat in Obesity and Diabetes Pharmacotherapy

Gianluca Iacobellis, Sara Basilico, and Alexis Elias Malavazos

Contents

Abstract

Epicardial adipose tissue surrounds and infiltrates the heart. Epicardial fat displays unique anatomic, genetic, and biomolecular properties. People with obesity and in particular, those with abdominal obesity and associated type 2 diabetes mellitus, have an increased amount of epicardial adipose tissue (EAT). Epicardial fat works well as therapeutic target due to its fast-responding

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metabolism, organ fat specificity, and easy measurability. Epicardial fat responds to thiazolidinediones (TZD), glucagon-like peptide 1-receptor agonists (GLP1A), sodium-glucose cotransporter 2 inhibitors (SGLT2i), dipeptidyl peptidase-4 inhibitors (DPP4i), and statins. Modulating epicardial fat morphology and genetic profile with targeted pharmacological agents suggests novel strategies in the pharmacotherapy of diabetes and obesity.

Keywords

Dipeptidyl peptidase-4 inhibitors · Epicardial adipose tissue: pharmaceutical target · Epicardial fat · Glucagon-like peptide 1-receptor agonists · Sodium glucose co-transporter 2 inhibitors · Statins · Thiazolidinediones

Key Points

- People with obesity and in particular, those with abdominal obesity and associated type 2 diabetes mellitus, have an increased amount of epicardial fat.
- Epicardial fat can serve as target for pharmaceutical agents targeting the adipose tissue.
- Glucagon-like peptide 1 analogs (GLP-1A) and sodium glucose co-transporter 2 inhibitors (SGLT2i) have shown to directly target and decrease excessive epicardial fat.

1 Introduction

Epicardial adipose tissue (EAT) is a peculiar visceral fat (Iacobellis et al. 2005, 2011; Iacobellis 2015). EAT lies between the myocardium and the epicardium including the epicardial coronary arteries. Differently, the pericardial fat is situated more externally. It is interesting to note that epicardial and intra-abdominal fat share the same embryogenesis as both evolve from brown fat. EAT is supplied by branches of the coronary arteries. In adults, epicardial fat can be mainly found in the atrioventricular and interventricular grooves. Under the microscope, epicardial fat displays a complexity of cells, mainly adipocytes as expected, but also neural, inflammatory, vascular, and immune cells. Epicardial fat should be considered a white adipose tissue. However, it presents with a number of brown-fat like or beige fat characteristics which become scarce with aging and advanced chronic diseases. One of the most unique features of EAT is its unobstructed conjunction with the myocardium that allows an anatomical and functional cross-talk throughout a shared local microcirculation. When the equilibrium between physiological function and pathological effects is lost, epicardial fat presents with a highly pro-inflammatory transcriptome and proteasome. Epicardial adipocytes are commonly infiltrated by macrophages and mast cells in subjects with diabetes and coronary artery disease.

With great and immediate advantages, EAT is measurable in the clinical practice with standard imaging techniques, such as ultrasound and computed tomography (Iacobellis et al. 2003b; Iacobellis and Willens 2009). EAT is undoubtedly a marker

of visceral adiposity (VAT), but not only. In addition to be an independent correlate of intra-abdominal fat, ultrasound-detected EAT reflects myocardial lipid content, measured with magnetic resonance (MR) spectroscopy (Iacobellis et al. 2003a; Malavazos et al. 2010). As myocardial lipid accumulation can lead to cardiac arrhythmias and disarray, EAT can serve as clinically measurable index of ectopic fat. However, a risk factor needs to be modifiable and targetable to be clinically useful. Recent discoveries and clinical trials have indicated that EAT can meet the criteria to be considered a therapeutic target in diabetes and obesity, two non-communicable diseases that are rapidly increasing in the global population (Saeedi et al. 2019; World Health Organization 2021). As an important cardiovascular risk factor in obesity and diabetes, EAT can, and should, be targeted and possibly modified throughout the use of several diabetes- and obesity-specific therapeutic strategies (Fig. 1). We herein will discuss the role of EAT as therapeutic target from bench to bedside. The effects on EAT by pharmaceutical drugs modulating the adipose tissue are summarized in Table 1.

2 EAT and Diabetes Mellitus

The prevalence of diabetes is concernedly rising with over 400 million of people diagnosed worldwide and a projected estimate of 700 million by 2045 (Saeedi et al. 2019).

Type 2 diabetes (T2D), the most prevalent subtype, is well known to be associated with an increased risk of cardiovascular disease (CVD) and mortality (Saeedi et al. 2020).

T2D is lately becoming more common in younger people and particularly in those with overweight and obesity, which exacerbates the risk of cardiovascular disease that is already high in patients with type 2 diabetes (Boudina and Abel 2010; Kim et al. 2016; Neeland et al. 2019).

T2D is associated with higher and abnormal EAT (Li et al. 2019). EAT has a peculiar transcriptome in subjects with diabetes (Camarena et al. 2017). Diabetic EAT is enriched with genes encoding for pro-inflammatory cytokines and growth factors involved in atherogenic pathways, such as Tumor Necrosis Factor (TNF), Nuclear Factor-κB (NF-κB), and advanced glycation end-products-receptor advanced glycation end products (AGE-RAGE). EAT inflammatory activity appears to be modulated by the AGE-RAGE signaling in subjects with diabetes. EAT is a metabolic active tissue with high lipolytic and secretory hyperactivity that can lead to accumulation of toxic lipid metabolites in the myocardium and endothelium that may accelerate atherosclerosis, cardiac remodeling, and heart failure in subjects with and without diabetes (Wende and Abel 2010; Tchkonia et al. 2013).

Notably, in patients with T2D, the fatty acid profile of EAT is different from those without T2D, with a decrease in palmitic acid (16:0) and omega-3 and an increase in trans fatty acids that worsen atheroma formation (Pezeshkian and Mahtabipour 2013).

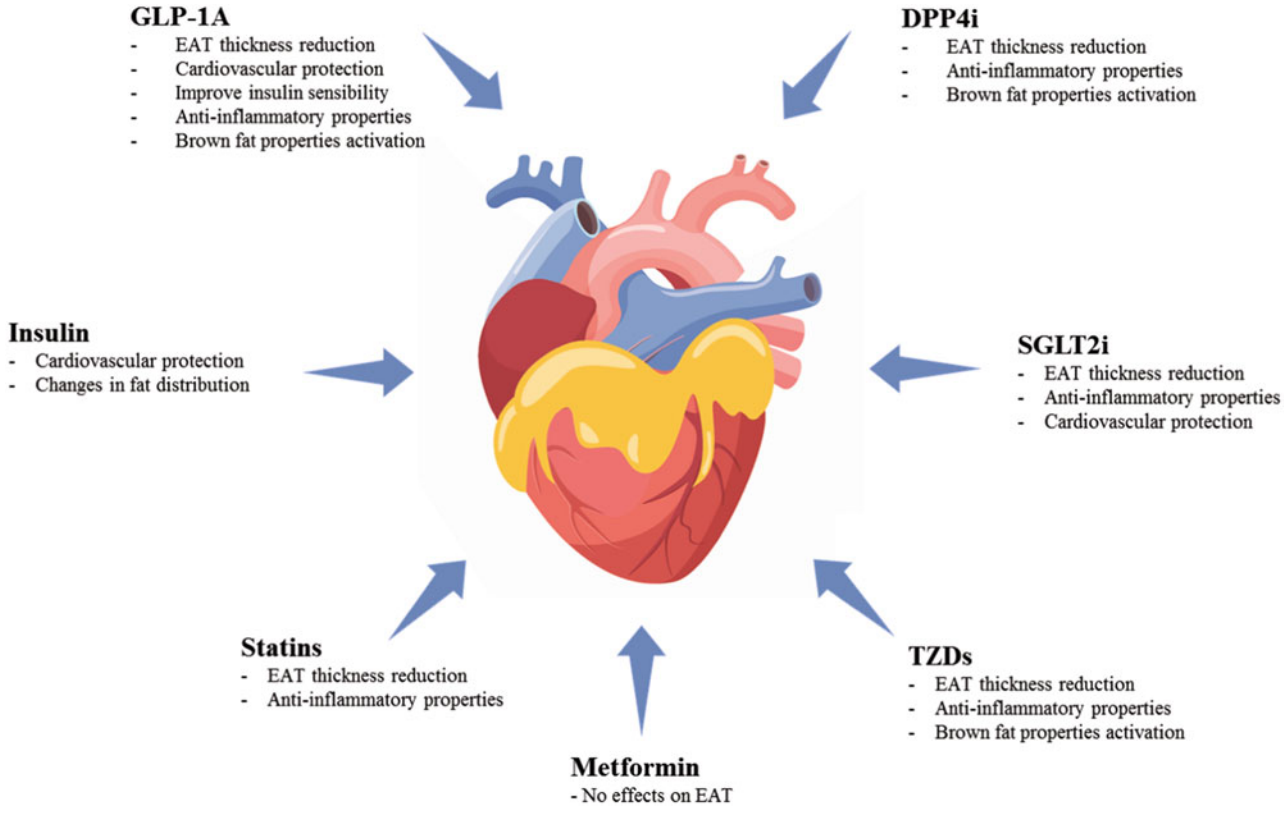

Fig. 1 Targeting epicardial fat in obesity and diabetes pharmacotherapy. *EAT* epicardial adipose tissue, *GPL-1A* glucagon-like peptide-1 receptor agonists, *SGLT2i* sodium glucose transporter 2 inhibitor, *DPP4i* dipeptidyl peptidase-4 inhibitor, *TZD* thiazolidinedione

Table 1 Pharmacotherapy effects on epicardial fat

Authors	Drug	Class	EAT change	Weeks
Iacobellis et al.	Liraglutide	GLP-1A	−42%	24
Morano et al.	Liraglutide or exenatide	GLP-1A	−13%	18
Iacobellis et al.	Semaglutide	GLP-1A	−20%	12
Iacobellis et al.	Dulaglutide	GLP-1A	−20%	12
Iacobellis et al.	Dapagliflozin	SGLT2i	−20%	24
Yagi et al.	Canagliflozin	SGLT2i	−20%	24
Fukuda et al.	Ipragliflozin	SGLT2i	−12%	12
Lima-Martinez et al.	Sitagliptin	DPP4i	−15%	24
Sacks et al.	Pioglitazone	TZD	–	
Park et al.	Atorvastatin	Statin	−10%	24
Alexopoulos et al.	Atorvastatin	Statin	−3%	48
Park et al.	Simvastatin	Statin	−3%	24
Alexopoulos et al.	Pravastatin	Statin	−0.8%	48

EAT epicardial adipose tissue, *GLP1RA* glucagon-like peptide 1 receptor agonist, *SGLT2i* sodium glucose transporter 2 inhibitor, *DPP4i* dipeptidyl peptidase-4 inhibitor, *TZD* thiazolidinedione

EAT secretosome from patients with T2D can affect cardiomyocyte contractility and fat oxidation (Greulich et al. 2012). Excessive EAT leads to upregulation of pro-inflammatory cytokines and down-regulation of anti-inflammatory molecules (Mazurek et al. 2003).

Overall, these metabolic alterations indicate that EAT has a pathophysiological potential that may be unique in subjects with diabetes. Diabetes if poorly controlled and accompanied by visceral obesity can potentiate the pro-inflammatory properties of EAT.

High EAT volumes, frequently observed in patients with T2D and obesity, can cause significant changes in left ventricle mass and volume and diastolic function (Iacobellis et al. 2011; Levelt et al. 2016; Christensen et al. 2019b).

EAT volume is also associated with high coronary artery calcium (CAC) score and severe coronary artery damage in patients with T2D (Wang et al. 2009; Yerramasu et al. 2012), although the role of EAT in CAD can be independent of CAC. EAT seems to be more commonly related to lipid-rich rather than calcified coronary plaques. This finding suggests the role of EAT in subclinical atherosclerosis or in the early phases of the disease, but in high risk patients. In this direction, EAT density, measured with computed tomography (CT), could provide an early marker of local inflammation (Iacobellis and Mahabadi 2019). EAT attenuation served as predictor of cardiometabolic risk also in the recently-emerged novel coronavirus-2019 pandemic (Malavazos et al. 2020).

In addition, in patients with T2D, a possible gender-specific role of EAT as a possible predictor of CVD was also reported (Christensen et al. 2019a).

Taken together, there is considerable evidence to suggest that EAT is associated with an increased risk of CVD especially in patients with T2D.

As an important cardiovascular risk factor in obesity and diabetes, EAT can and should be modified through the use of diabetes- and obesity-specific therapeutic strategies and lifestyle changes.

3 EAT and Obesity

Obesity is a complex and multifaceted condition (Jastreboff et al. 2019). Obesity, defined as body mass index (BMI) $\geq$30 kg/m^2, is linked to higher cardiovascular mortality when compared to normal weight. BMI-defined obesity is well known to be associated with insulin resistance, low-grade chronic inflammation, hypertriglyceridemia, T2D, atherosclerosis, and major cardiovascular events (Flegal et al. 2005; Dallongeville et al. 2012).

However, BMI is not the most accurate index of excess adiposity because it does not reflect the distribution of body fat, which can substantially vary among people with the same BMI scores (Okorodudu et al. 2010). Location is the actual key when it comes to body fat. Studies have been then moving the focus to the intra-abdominal fat rather than BMI only. Studies using CT or Magnetic Resonance Imaging (MRI) indicated that abdominal fat depots are very heterogeneous and differentially associated with atherosclerosis and cardiometabolic risk (Fox et al. 2007; Neeland et al. 2013). Intra-abdominal visceral fat (VAT), which surrounds and infiltrate inner organs, is now considered an independent marker of morbidity and mortality, whereas intra-abdominal subcutaneous adipose tissue (SAT) is a weaker indicator of cardiovascular risk (Hiuge-Shimizu et al. 2012). There are several biomolecular reasons why VAT is associated with higher cardiovascular risk than SAT.

One of the mechanisms that has been evoked is the inadequateness of SAT to expand in response to excess triglycerides. On the contrary, SAT would act as a protective metabolic sink in the context of a positive energy balance results in lipid deposition in tissues that are not deputed for adipose storage. This results in the accumulation of lipids in lean tissues such as the liver, heart, and skeletal muscle, a process known as ectopic fat deposition (Smith and Kahn 2016). However, some visceral fat depots seem to have intrinsic high risk genetic and biochemical properties, regardless of the ectopic fat redistribution. This can apply to EAT, a VAT with peculiar and unique transcriptome and proteasome. Hence, the focus has been narrowed to organ-specific visceral fat depots, rather than intra-abdominal VAT only. The excess of EAT is associated with increased VAT deposition and higher risk of developing atherosclerosis and coronary artery disease (Iacobellis et al. 2003a, b; Malavazos et al. 2008). Since CT and MRI are mostly used for research purposes and may be expensive or not readily available in hospital or clinical setting, several anthropometric indices of visceral fat are used as surrogate markers of VAT. The most widely used and accepted among these measurements is waist circumference. In particular, the cutoff for the assessment of visceral obesity is defined by waist circumference $\geq$ 102 cm for males and $\geq$ 88 cm for females (Grundy et al. 2005). The combined presence of an increased waist circumference and high triglyceride levels has been associated with a high probability (around 80%) of increased VAT levels (Lemieux et al. 2000). This condition is called

hyper-triglyceridemic waist phenotype and represents a simple tool to screen for the presence of excess VAT and ectopic fat (Lemieux et al. 2007). Nevertheless, waist circumference is an operator-dependent index and biased by a large variability. Given this poor reproducibility and accurateness, the need for easier and more reproducible imaging marker of VAT was compelling. The ultrasound measurement of EAT thickness, first proposed and validated by Iacobellis (Iacobellis et al. 2003a) as marker of VAT, may meet some of these criteria, although ultrasound has some limitations, too.

VAT is certainly a key conduit through which obesity predicts health risk. Body fat distribution is so more important than general obesity that it is common to encounter severely BMI-defined subjects with obesity with no cardiometabolic complications. These metabolically healthy, but obese individuals tend to have more SAT than VAT. Excess visceral adiposity is characterized by increased visceral and ectopic fat deposition, adipocyte dysfunction, inflammatory and adipokine dysregulation, and insulin resistance. The expanded VAT becomes highly inflamed with dense infiltration of macrophages within hypertrophied adipocytes (Mazurek et al. 2003; Iacobellis et al. 2005; Ouchi et al. 2011; Iacobellis 2015).

EAT becomes thicker and dysfunctional, causing cardiovascular damages through alterations of cardiac morphology and function, such as increased left ventricular mass and diastolic dysfunction (Rabkin 2014). Larger amount of EAT is characterized by an increased uptake of free fatty acids (FFAs), adipocyte hypertrophy, inability to store triglycerides, increased lipolysis, and inflammation (Rabkin 2014). In addition, EAT can release FFAs in the coronary arteries modulating vascular responsiveness to vasoactive agents (Henrichot et al. 2005). The secretion of epicardial pro-inflammatory cytokines into the coronary bloodstream and the increased infiltration of inflammatory macrophages promote atherogenesis, as plaque accumulation tends to increase the arterial wall thickness (Iacobellis 2015) and cause plaque rupture leading to amplification of vascular inflammation and plaque instability via apoptosis and neovascularization (Sacks and Fain 2007). In patients with obesity, anti-inflammatory cytokines decrease, contributing to the risk of cardiometabolic diseases. Thus, in patients with obesity EAT may become an adverse pro-inflammatory organ and can be considered a risk factor for metabolic and cardiovascular diseases (Aslanabadi et al. 2014). Therefore, EAT thickness and volume are considered surrogates for metabolically unhealthy abdominal obesity and have been used for cardiovascular risk stratification.

A major goal in the therapeutic field of abdominal obesity and related cardiovascular disorders is the development of effective treatments to reduce VAT and EAT.

4 EAT and GLP-1A

Glucagon-Like Peptide-1 Receptors Agonists (GLP-1A) are anti-diabetic medications with potential beneficial effects that can go beyond the glycemic control. Clinically significant weight loss is commonly observed in patients treated with GLP-1A (Marso et al. 2016a, b; Gerstein et al. 2019). Recent trials also showed

that GLP-1 receptor agonists reduced major adverse cardiovascular events (Marso et al. 2016a, b; Gerstein et al. 2019). Liraglutide, a daily GLP-1 receptor agonist, has been associated with weight loss in type 2 diabetic patients and lower risk of cardiovascular events (Marso et al. 2016a). Weekly GLP-1 receptor agonists, semaglutide and dulaglutide have been also correlated with weight loss and lower mortality for cardiovascular events (Gerstein et al. 2019).

The effect GLP-1A on EAT was only recently evaluated. In a 24-week interventional case-controlled study in overweight/obese type 2 diabetic, liraglutide added on metformin reduced EAT thickness from 9.6 ± 2.0 to 6.8 ± 1.5 and 6.2 ± 1.5 mm ($p < 0.001$) after 12 and 24 weeks, respectively, accounting for a 36% of reduction at 24 weeks, whereas there was no significant EAT reduction in the Metformin group. (Iacobellis et al. 2017b). In another study, liraglutide or exenatide, a weekly GLP-1 receptor agonist, caused a smaller reduction of EAT thickness after 12 weeks of treatment (Morano et al. 2015). Exenatide reduced ectopic fat infiltration also in the heart and liver (Dutour et al. 2016).

More recently, the EAT modulation of new generation long-acting, weekly GLP-1A, semaglutide and dulaglutide was investigated. In a 12-week, controlled, parallel study in a cohort of patients with T2D and obesity both semaglutide or dulaglutide induced a rapid and dose-dependent reduction in EAT thickness, (Iacobellis and Villasante Fricke 2020). Ultrasound measured EAT thickness significantly decreased in both semaglutide and dulaglutide groups ($p < 0.001$) after 12 weeks, accounting for a 20% reduction. However, EAT thickness decrease was greater ($p < 0.01$) with the higher doses of semaglutide (1 mg) and dulaglutide (1.5 mg), respectively.

Is GLP-1A effect on EAT specific or mediated by overall weight loss? Our group showed that human EAT, collected from patients undergoing elective cardiac surgery, expressed GLP-1 receptors (GLP-1R) (Iacobellis et al. 2017a). RNA-sequencing analysis revealed that EAT expresses GLP-1R genes and immunofluorescence confirmed the presence of GLP1R protein within EAT whereas the signal was absent in the SAT sample obtained from the same patient.

Based on these findings GLP-1 receptor agonists effects may specifically target EAT, although the role of the overall weight loss cannot be ruled out. The mechanisms leading to the fat reduction in response to the GLP-1R activation are unclear. GLP-1 can promote EAT preadipocyte differentiation, improve insulin sensitivity, and stimulate EAT thermogenesis and adipocyte browning (Pyke and Knudsen 2013; Yang et al. 2013; Beiroa et al. 2014). GLP-1 can promote preadipocyte differentiation and then improve insulin resistance. Both lipolytic and lipogenic dose-dependent effects of GLP-1 were described (Vendrell et al. 2011). EAT GLP-1R was directly correlated with genes promoting beta-oxidation and white-to-brown adipocyte differentiation, and inversely with pro-adipogenic genes (Dozio et al. 2019). GLP-1 analogs may target EAT GLP-1R and therefore reduce local adipogenesis, improve fat utilization, and induce brown fat differentiation. GLP-1 induced browning effect on EAT is an interesting mechanism that may warrant future investigations to be confirmed.

5 EAT and DPP4i

Dipeptidyl peptidase-4 inhibitors (DPP-4i) are another class of medications indicated for the treatment of T2D that inhibit the degradation of GLP-1. In a single clinical study in overweight/obese individuals with T2D who were not well controlled on metformin monotherapy, the addition of sitagliptin, one of the most commonly used DPP4is, produced a 15% reduction of EAT (Lima-Martínez et al. 2016). This effect is likely driven by the activation of the GLP-1R, and the prolonged half-life of GLP-1 mediated by the inhibition of DPP-4 may have an important effect on the EAT reduction. DDP4 inhibition could also reduce EAT inflammation (Shah et al. 2011; Aroor et al. 2013) and increase the brown fat activators (Shimasaki et al. 2013).

6 EAT and SGLT2i

Selective sodium-glucose cotransporter 2 inhibitors (SGLT2is) are relatively novel oral anti-diabetic agents. Patients commonly lose weight with SGLT2is. Although the mechanisms are not fully understood, yet, SGLT2i-related weight loss could result from caloric loss or fluid loss secondary to osmotic diuresis or from a combination of both factors. The EMPA-REG OUTCOME, CANVAS, and DECLARE-TIMI 58 trials recently showed that SGLT2i reduce major adverse cardiovascular events mainly in patients with established atherosclerotic cardiovascular disease (Zinman et al. 2015; Mahaffey et al. 2018; Wiviott et al. 2019).

Our group recently evaluated the effects of dapagliflozin, a commonly used SGLT2i (Bolinder et al. 2012; Guedes et al. 2013) on EAT in a 24-week randomized double-blind placebo controlled clinical trial (Iacobellis et al. 2020). In patients who were randomized to dapagliflozin, ultrasound-measured EAT thickness decreased by 20% from baseline to 24 weeks ($p < 0.01$r all), whereas in the metformin group there was a significant, but smaller EAT reduction (Iacobellis et al. 2020). This randomized clinical trial suggested that dapagliflozin caused a rapid and significant EAT reduction, partially independent of weight loss. Another study showed that 6-month therapy with dapagliflozin reduced EAT volume and inflammatory markers (Sato et al. 2018). What is the mechanism behind the effects of dapagliflozin on EAT? To address this question, SGLT2 expression was analyzed by real-time polymerase chain reaction Western blot, and immunohistochemistry from fat samples obtained from patients undergoing cardiac surgery (Díaz-Rodríguez et al. 2018). Fat explants were then treated with dapagliflozin and/or insulin. Dapagliflozin up-regulated EAT glucose uptake, down-regulated the secretion of pro-inflammatory adipokines, and facilitated the differentiation of epicardial adipocytes (Díaz-Rodríguez et al. 2018).

Canagliflozin, another commonly prescribed SGLT2i, lowered EAT thickness after 24 weeks in patients with type 2 diabetes (Yagi et al. 2017). Epicardial fat volume decreased also in patients treated with two other recently developed SGLT2i, ipragliflozin and luseogliflozin (Bouchi et al. 2017; Fukuda et al. 2017).

Overall, SGLT2i can produce changes in EAT mass and bio-molecular properties, although the exact mechanisms for these effects are still unknown. It is unclear if the effect is mediated by the weight loss. Further studies are needed to better evaluate the independent effects of SGLT2i on EAT.

7 EAT and TZDs

Thiazolidinediones (TZDs), also known as glitazones are agonists of the peroxisome proliferator-activated receptors (PPARs). TZD can lower blood glucose, improve insulin sensitivity, lipid metabolism and change body fat distribution. TZDs are associated with body fat redistribution with VAT reduction and a compensatory increase in SAT.

Pioglitazone, a commonly used TZD, can down-regulate EAT pro-inflammatory transcriptome in subjects with T2D and coronary artery disease (Sacks et al. 2011). Subjects who were treated with pioglitazone had a lower expression of EAT interleukin-1β and other genes involved in inflammation and atherosclerosis. It is interesting to note that PPAR-γ agonist can induce a rapid browning of the epicardial fat in experimental models. Pioglitazone effects on EAT mass are controversial. One study showed a significant reduction of EAT thickness in T2D subjects (Nagai et al. 2008). This finding was not confirmed in a 24-week prospective, double-blind, randomized, controlled study comparing pioglitazone with metformin (Jonker et al. 2010). Nevertheless this study measured pericardial rather than epicardial fat.

Rosiglitazone up-regulated PPAR-γ coactivator 1 alpha (PGC1-α), a key precursor of brown fat, in epicardial adipocytes of Zucker rats (Distel et al. 2012). Thiazolidinediones may therefore resume or activate EAT brown fat properties, although this hypothetical mechanism would need to be evaluated in humans. Pharmaceutical targeting epicardial fat with TZDs in patients with high cardiovascular risk may result in reduced inflammation and improved metabolic profile through a PPARs stimulation. HIV positive patients who develop metabolic syndrome may particularly benefit by use of TZDs targeting EAT (Iacobellis et al. 2007a, b).

8 EAT and Metformin

There are no randomized controlled trials that examined the independent effect of metformin on EAT, although a number epicardial fat studies used metformin as control treatment. Very few studies found significant changes in EAT thickness in patients added-on or started on metformin monotherapy (Iacobellis et al. 2020). As today, it is reasonable to say that metformin has a no or minimal effects on EAT.

9 EAT and Insulin

It is known that long-acting insulin analogs provide beneficial cardiovascular effects. However, long-acting insulin can also cause minor or more significant changes in body weight and fat distribution. A 24-week interventional study compared the effects of detemir vs glargine on EAT thickness in insulin-naïve inadequately controlled patients with type 2 diabetes (Elisha et al. 2016). In patients who received detemir, EAT thickness changes correlated with truncal fat and total fat mass changes. Detemir resulted in less fat mass gain, a trend for a more pronounced EAT thickness reduction when compared with glargine. However, we can conclude that EAT is unlikely a sensitive target to insulin.

10 EAT and Statins

HMG-CoA reductase pathway inhibitors, commonly known as statins, have multiple effects including modulation and reduction of adipose tissue inflammation. EAT thickness decreased by 10% in patients receiving atorvastatin vs. 3.1% in those treated with simvastatin/ezetimibe (Park et al. 2010). Consistently, atorvastatin reduced EAT volume in post-menopausal women (Alexopoulos et al. 2013), independently of lipid lowering or coronary artery disease progression. Interestingly, pioglitazone, simvastatin, or combination treatment reduced EAT inflammatory markers in patients with coronary artery disease (Grosso et al. 2014). EAT PPAR receptors could be the target of both pioglitazone and simvastatin. Interestingly, adipocyte metabolism requires activation of the PPARγ, which is upregulated in human embryonic ventricular epicardial cells (Yamaguchi et al. 2015).

11 Conclusions

When it comes to stratification and prevention of the cardiovascular risk in subjects with diabetes and obesity, the need of a measurable biomarker and modifiable target to track changes during pharmaceutical interventions is compelling. EAT can meet all criteria to be a good and traceable imaging marker. Its rapid and substantial responsiveness to drugs displaying pleiotropic effects such GLP-1A and SGLT2i appears very promising and with immediate application in the clinical practice. It warrants future studies the hypothesis to not only reduce EAT mass, but also to modulate EAT function to resume its original cardio-protective properties.

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The Enteroendocrine System in Obesity

Emily L. Miedzybrodzka, Frank Reimann, and Fiona M. Gribble

Contents

Abstract

The enteroendocrine system coordinates the physiological response to food intake by regulating rates of digestion, nutrient absorption, insulin secretion, satiation and satiety. Gut hormones with important anorexigenic and/or insulinotropic roles include glucagon-like peptide 1 (GLP-1), peptide YY ($PYY_{3–36}$), cholecystokinin (CCK) and glucose-dependent insulinotropic peptide

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(GIP). High BMI or obesogenic diets do not markedly disrupt this enteroendocrine system, which represents a critical target for inducing weight loss and treating co-morbidities in individuals with obesity.

Keywords

CCK · Diabetes · Enteroendocrine · GIP · GLP-1 · Gut hormone · Obesity · PYY

1 Introduction

Enteroendocrine cells (EECs) secrete around 20 active peptides in response to ingested nutrients, neurohormonal signals and luminal contents such as bile acids. This enteroendocrine system makes the gastrointestinal (GI) tract the body's largest endocrine organ (Ahlman and Nilsson 2001). Unlike other hormones typically secreted from large specialised endocrine glands, enteroendocrine cells are scattered throughout the GI epithelium, making up only around 1% of the total cell number. These gut peptide hormones play concerted physiological roles in the control of digestion, absorption, nutrient availability and satiety. Each gut hormone has multiple physiological functions, and most functions can be performed by several hormones. This apparent redundancy enables coordination of digestive and absorptive processes, while also contributing to postprandial and long-term energy homeostasis. This chapter will focus on the regulation of food intake, insulin secretion and GI motility by several key enteroendocrine hormones, and how this axis may be dysregulated in obesity.

Hormone expression differs significantly along the length of the gastrointestinal tract, reflecting the variety of stimuli to which cells are exposed. In the proximal small intestine EECs secrete hormones rapidly upon absorption of digested nutrients, thus enabling peripheral tissues such as the pancreas to prepare for the forthcoming entry of nutrients into the circulation. Distal EECs of the ileum and colon, which are rarely exposed to ingested nutrients, release hormones in response to a variety of neurohormonal stimuli and microbial products. Intestinal EECs are polarised – hormones are secreted basolaterally into the interstitial space, and apical 'open-type' processes extend into the gut lumen (Eissele et al. 1992). It was initially assumed that these apical processes 'sample' the luminal content; however, it has been demonstrated that most stimulus detection occurs at the basolateral cell surface (Brighton et al. 2015; Christensen et al. 2015). This suggests that local absorption of nutrients, rather than simply their presence in the lumen, is the key cue for hormone release.

Secreted gut hormones enter the peripheral circulation and exert their endocrine actions through G protein coupled receptors (GPCRs) located on target tissues. Gut hormones can also act locally on absorptive enterocytes, other EECs, and enteric or peripheral neurons. EECs additionally secrete several small molecule neurotransmitters which can sensitise hormonal responses or directly trigger action

potentials in sensory neurons, including glutamate (Kaelberer et al. 2018), ATP (Lu et al. 2019) and serotonin (Bellono et al. 2017).

A number of enteroendocrine hormones and their receptors have been implicated in the central control of food intake. GPCRs for several hormones are expressed in vagal afferent neurons, enabling direct signalling from the gut to the central nervous system (CNS) via the nodose ganglia (Wang et al. 2020). Despite the short half-life of circulating gut hormones, evidence suggests that many are also able to act via receptors located in the brainstem and hypothalamus. Several of the so-called gut hormones are also expressed by neurons and glial cells within the CNS, which may represent the endogenous source of ligands for gut hormone receptors situated behind the blood-brain barrier (Woodward et al. 2021).

Hormones with reported anorexigenic actions include glucagon-like peptide 1 (GLP-1) and other proglucagon-derived (*GCG*) peptides, glucose-dependent insulinotropic polypeptide (GIP), peptide YY (PYY) and cholecystokinin (CCK). The appetite-suppressive and other beneficial metabolic effects of these hormones present promising targets for the treatment of type 2 diabetes and, increasingly, obesity.

2 Glucagon-Like Peptide 1 (GLP-1)

2.1 Intestinal GLP-1 Secretion

Secretion of GLP-1 from enteroendocrine L-cells in the small intestine is strongly stimulated by products of nutrient digestion. Consumption of carbohydrates, and to a lesser extent protein, induces rapid GLP-1 secretion which peaks after 30–60 min (Elliott et al. 1993; Herrmann et al. 1995). Fat also evokes significant GLP-1 release, on a slower timescale (Elliott et al. 1993). There is a rapid early rise in GLP-1 secretion after oral glucose ingestion, detectable in the plasma within 5 min (Herrmann et al. 1995). While the majority of L-cells are situated in the ileum and colon, selective ablation of *Gcg* expression in the distal gut has demonstrated that the relatively sparse population of duodenal and jejunal L-cells can account for much of the early GLP-1 secretory response (Panaro et al. 2020; Song et al. 2019).

GLP-1 secretion can be sustained for over 2 h following a meal, depending on its macronutrient composition, reflecting the prolonged activation of L-cells in the distal gut (Elliott et al. 1993). Bile acids, which emulsify lipids to aid digestion, also evoke significant GLP-1 secretion and likely contribute to the strong, sustained secretory response to ingested fats (Brighton et al. 2015; Goldspink et al. 2018; Thomas et al. 2009). In the colon, where L-cells are rarely exposed to ingested nutrients, several microbial metabolites act as strong stimuli of GLP-1 release – including short-chain fatty acids (SCFAs), modified secondary bile acids, lipopolysaccharide and indole (Chimerel et al. 2014; Lebrun et al. 2017; Panaro et al. 2020; Tolhurst et al. 2012).

Neurohormonal signals regulate GLP-1 release both postprandially and in the fasting state. For example, somatostatin (SST), a general inhibitory hormone released from enteroendocrine D-cells throughout the GI tract, exerts paracrine

suppression of GLP-1 secretion (Hansen et al. 2000; Jepsen et al. 2019). L-cells also respond to endocrine signals from other organs, such as angiotensin II and arginine vasopressin (Pais et al. 2016a, b). Several enteric or vagal neurotransmitters stimulate GLP-1 release, such as acetylcholine (Balks et al. 1997) and gastrin-releasing peptide (Roberge et al. 1996), while the neuropeptide galanin suppresses GLP-1 secretion (Herrmann-Rinke et al. 1996; Psichas et al. 2016).

2.2 GLP-1 and the Incretin Effect

Oral or intrajejunal delivery of glucose causes a much greater increase in insulin secretion than isoglycaemic intravenous glucose, implicating some intestinal-derived factor (McIntyre et al. 1964). GLP-1 and its sister hormone GIP underlie this so-called incretin effect, which accounts for around two-thirds of insulin secretion after glucose ingestion (Dupre et al. 1973; Kreymann et al. 1987; Perley and Kipnis 1967). GLP-1 directly activates the Gs-coupled GLP-1 receptor (GLP1R) on β-cells, sensitising the cellular machinery to glucose-induced Ca^{2+} signals. Pancreatic α-cell glucagon secretion is in contrast suppressed by GLP-1; this further contributes to its glucose-lowering effects through a reduction of hepatic glycogenolysis and gluconeogenesis (Hare et al. 2010; Orskov et al. 1988). As the GLP1R is only expressed in around 10% of α-cells, this is likely an indirect effect achieved via release of inhibitory SST from pancreatic δ-cells (de Heer et al. 2008; Richards et al. 2014). Beyond the acute endocrine pancreatic effects of GLP-1, it has also been proposed to increase β-cell mass (at least in rodents), promote insulin synthesis, and increase exocrine pancreatic secretions (Drucker et al. 1987; Hou et al. 2016; Li et al. 2005). The insulinotropic and glucagonostatic actions of GLP-1 made it a key target for the development of anti-diabetic medications and would also be useful in obese individuals with impaired glucose tolerance.

2.3 Anorexigenic Effects of GLP-1

Early studies demonstrated that central administration of native GLP-1 to rats reduced acute food intake and body weight (Meeran et al. 1999; Turton et al. 1996), while peripheral GLP-1 enhanced satiety and decreased energy intake in humans (Gutzwiller et al. 1999). Notably, GLP-1 mimetics used for the clinical treatment of T2DM induce profound weight loss (Drucker and Nauck 2006). Circulating GLP-1 and exogenous agonists can directly activate GLP1R located on central neurons in regions with a relatively permeable blood-brain barrier, including the median eminence, in close proximity to hypothalamic nuclei implicated in feeding regulation such as the arcuate and paraventricular nuclei, and area postrema of the brainstem adjacent to the nucleus of the solitary tract (Gabery et al. 2020; Orskov et al. 1996; Richards et al. 2014; Yamamoto et al. 2003). The half-life of GLP-1 is extremely short (1–2 min) due to rapid inactivation by dipeptidyl peptidase 4 (DPP4) and renal clearance (Deacon et al. 1995; Hansen

et al. 1999). Some argue that circulating levels of active GLP-1 are therefore unlikely to reach meaningful concentrations, leading to suggestions that peripheral afferent signalling from the intestine may be more physiologically relevant for the anorexigenic effects of GLP-1 (Williams et al. 2009). GLP1R is expressed in cell bodies of the vagal nodose ganglia neurons which also respond to gastrointestinal stretch (Richards et al. 2014; Williams et al. 2016). Viral-mediated *Glp1r* knockdown in nodose ganglia increased meal size but did not alter long-term energy balance, suggesting that vagal afferent signalling may be primarily important for acute postprandial effects of GLP-1 (Krieger et al. 2016). Arguing for at least partial central action of peripherally administered pharmacological GLP1R agonists, the weight loss and food intake effects of liraglutide are maintained in mice with selective *Glp1r* reduction in *Phox2b*-expressing peripheral nerves, including vagal afferents, but lost following central *Glp1r* knockdown (Sisley et al. 2014).

Despite the anorexigenic effects of GLP1R agonists, gut-selective *Gcg* knockout does not alter body weight or acute food intake in mice (Song et al. 2019) and GLP1R antagonism with peripheral exendin-9 only has small effects on appetite (Steinert et al. 2014; Svane et al. 2016), questioning the importance of physiological levels of gut-derived GLP-1 for appetite control. GLP-1 is also generated within the CNS, where it can act as a neurotransmitter (Barrera et al. 2011). Preproglucagon (PPG) neurons in the nucleus tractus solitarius of the brainstem project to GLP1R-expressing hypothalamic nuclei (Llewellyn-Smith et al. 2011; Richards et al. 2014). These neurons act independently of gut-derived GLP-1 or GLP1R agonists to evoke hypophagia when activated by gastric distension, CCK or leptin – signals linked to energy balance (Brierley et al. 2021; Hisadome et al. 2010, 2011; Vrang et al. 2003). While PPG neurons are important for the control of food intake under conditions of psychological or homeostatic stress, loss of these neurons does not evoke overeating in ad libitum fed rodent models (Barrera et al. 2011; Holt et al. 2019). GLP1R agonists used pharmacologically directly activate several central GLP1R-expressing neural populations, and there is a possibility of further enhancing their anorexigenic effects in the treatment of obesity by manipulation of the PPG neural circuitry (Brierley et al. 2021; Gabery et al. 2020).

2.4 Gastrointestinal Effects of GLP-1

Gut-derived GLP-1 is an important regulator of gastrointestinal motility and function to modulate postprandial digestion and absorption (Song et al. 2019). This ‘ileal brake’ involves strong suppression of gastric emptying by GLP-1 and PYY released from the distal small intestine, to limit further nutrient delivery (Wettergren et al. 1993). These effects are mediated by vagal afferent signalling as they are lost following vagotomy, vagal nerve denervation or localised knockdown of GLP1R in vagal afferent neurons (Imeryuz et al. 1997; Krieger et al. 2016; Wettergren et al. 1997). GLP-1 also inhibits gastric acid secretion (Schjoldager et al. 1989) and reduces intestinal motility (Tolessa et al. 1998). Overall, this results in a slowing

of nutrient passage and distension of the gastrointestinal wall, signalling to satiety centres via stretch-activated afferent neurons (Powley and Phillips 2004).

2.5 Additional Actions of GLP-1

Several other, largely beneficial, direct or indirect effects of endogenous and pharmacological GLP-1 have been reported and validated to differing degrees. These include improved cardiac function, enhanced bone remodelling, reduced liver gluconeogenesis and steatosis, increased brown adipose tissue thermogenesis, reduced fluid intake, increased renal sodium and urine excretion, and neuroprotection [recently reviewed in Muller et al. (2019)].

3 Other Intestinal Proglucagon-Derived Peptides

The proglucagon gene (*GCG*) is post-translationally processed to form different peptides depending on the tissue-specific expression of prohormone convertase (PC) enzymes. Glucagon is produced in pancreatic α-cells by PC2, whereas in the intestine and central nervous system PC1/3 generates GLP-1, GLP-2, oxyntomodulin and glicentin (Mojsov et al. 1986; Muller et al. 2019). GLP-2 and oxyntomodulin are also secreted from L-cells and have relevant metabolic roles.

3.1 GLP-2

GLP-2 acts locally to promote intestinal regeneration and repair of the epithelial barrier, via the Gs-coupled receptor GLP2R (Benjamin et al. 2000; Drucker et al. 1996). The GLP-2 analogue teduglutide is used clinically in the treatment of short bowel syndrome (Burness and McCormack 2013). There are some reports that GLP-2 may promote glucagon secretion (Bahrami et al. 2010), enhance hepatic insulin sensitivity (Shi et al. 2013), improve glucose homeostasis (Baldassano et al. 2015), inhibit food intake (Baldassano et al. 2012) and reduce neuroinflammation (Nuzzo et al. 2019) in mice, particularly in diet-induced or genetic obesity models. There have been limited studies on the role of GLP-2 in humans beyond its intestinotrophic effects and, while physiological concentrations do not alter food intake, there is some evidence of GLP-2-mediated glucagon secretion (Lund et al. 2011; Sorensen et al. 2003).

3.2 Oxyntomodulin

In humans with obesity, oxyntomodulin increases energy expenditure and suppresses appetite to induce weight loss (Wynne et al. 2006) while also improving glucose tolerance (Shankar et al. 2018). No unique receptor for oxyntomodulin has

been identified to date but these effects have been shown to act via weak dual agonist activity at the GLP-1 and glucagon receptors (Baggio et al. 2004).

4 Glucose-Dependent Insulinotropic Polypeptide (GIP)

GIP release from proximal small intestinal K-cells is rapidly stimulated in response to intake of carbohydrates, fats and proteins (Lu et al. 2021). The insulinotropic incretin effect of GLP-1 is complemented by GIP, which activates its Gs-coupled receptor GIPR to engage overlapping signalling pathways in β-cells (Vilsboll et al. 2003). Because GIP infusion does not improve glucose tolerance in diabetic patients, likely due to concomitant stimulation of glucagon secretion (Chia et al. 2009; Nauck et al. 1993), it has been historically marginalised as a candidate therapeutic option for obesity and T2DM.

Loss of the GIPR is linked to reduced adiposity in high-fat fed or leptin deficient mice (Boer et al. 2021; Miyawaki et al. 2002); however, results of K-cell GIP ablation are contradictory (Holst et al. 2016; Nasteska et al. 2014) and chronic elevation of GIP in mice reduces the extent of diet-induced obesity (DIO) (Kim et al. 2012). In humans, GIP increases blood flood to the adipose tissue and while high fasting GIP levels are correlated with lower serum low-density lipoprotein concentrations, they are also associated with a less healthy distribution of fat depots in males (Asmar et al. 2010; Moller et al. 2016). Despite these proposed links to adiposity, GIPR agonism has been shown to potentiate the anorexigenic effects of GLP1R agonists in humans and mice (Finan et al. 2013). These results remain controversial and represent an active area of study, but the potent weight loss observed with the GIPR/GLP1R dual agonist tirzepatide currently under development makes GIP a key target in new treatments for obesity (Frias et al. 2018).

In addition to its metabolic effects, GIP inhibits bone resorption in mice and humans to promote bone strength (Nissen et al. 2014; Xie et al. 2005). This may underlie a genetic association of GIPR mutation to fracture risk in post-menopausal women (Torekov et al. 2014) and GIPR agonist treatment could theoretically reduce osteoporosis in patients.

5 Peptide YY (PYY)

PYY is localised with GLP-1 in L-cells and is co-secreted from the same vesicles (Billing et al. 2018; Nilsson et al. 1991). Prevalence of PYY is higher in more distal regions of the intestinal tract compared with GLP-1, underlying the greater stimulation of PYY secretion in response to ingested lipids versus carbohydrates (Habib et al. 2012; Steinert et al. 2017). The secreted form $PYY_{1–36}$ can activate all members of the Gi-coupled neuropeptide Y receptor (NPYR) family at different potencies; subsequent cleavage by DPP4 generates $PYY_{3–36}$, which exerts biological activity primarily via the NPY2R (Ballantyne 2006).

Exogenous $PYY_{3–36}$ reduces food intake in humans (Batterham et al. 2002; Degen et al. 2005). Central administration of an NPY2R antagonist is sufficient to block the effects of $PYY_{3–36}$ on food intake (Abbott et al. 2005b), although evidence on the impact of vagotomy and contribution of peripheral NPY2R-expressing neurons is inconsistent (Abbott et al. 2005a; Halatchev and Cone 2005; Koda et al. 2005). The anorexigenic effects of distal colon L-cell activation are blocked by NPY2R inhibition (Lewis et al. 2020), lending support to earlier suggestions that $PYY_{3–36}$ may be more physiologically important than GLP-1 in evoking satiety (Steinert et al. 2014).

Like GLP-1, both $PYY_{1–36}$ and $PYY_{3–36}$ contribute to the 'ileal brake' – inhibiting gastric emptying, slowing GI motility and reducing gastric acid secretion (Chelikani et al. 2004; Eissele et al. 1990; Savage et al. 1987). A further key role of $PYY_{1–36}$ is the NPY1R-mediated reduction of fluid secretion from colonic enterocytes into the lumen, thus contributing to osmoregulation downstream of angiotensin II (Pais et al. 2016a). PYY represents a critical satiety hormone which likely underlies much of the effect of bariatric surgery, and PYY mimetics are at early stages of clinical development for obesity.

6 Cholecystokinin (CCK)

CCK, primarily secreted from the proximal small intestine in response to fats and protein, was first identified for its role in inducing contraction of the gallbladder and relaxation of the sphincter of Oddi to release bile into the duodenum (Ivy and Oldberg 1928; Shaffer 1982; Toouli et al. 1982). Other gastrointestinal roles of CCK include inhibition of gastric emptying, stimulation of exocrine pancreatic secretions and SST-dependent reduction of gastric acid secretion (Fried et al. 1991; Kanagawa et al. 2002; Liang et al. 2017).

In humans, both exogenous and endogenous CCK reduce meal size (Ballinger and Clark 1994; Kissileff et al. 1981), an effect which is blocked by the CCK1 receptor antagonist loxiglumide (Lieverse et al. 1994). Both central and vagal CCK1Rs are important for these satiety effects in animal models (Reidelberger et al. 2004). Like GLP-1, CCK peptides are established neurotransmitters in the central nervous system (Rehfeld 2017). It is therefore critical that any anti-obesity drugs targeting the CCK axis are selective for CCK1R, as activation of the CCK2R induces severe neurological side effects including panic attacks (Bradwejn and Koszycki 2001).

7 Dysregulation of Gut Hormones in Obesity

It remains unclear how and to what extent diet or metabolic status affect enteroendocrine cell number and expression profiles. Several studies have demonstrated associations between diet, body mass index (BMI) and circulating levels of gastrointestinal hormones; however, the direction of effect has been

inconsistent. As outcomes differ between studies, it remains a challenge to delineate the effects of altered adiposity, insulin resistance, microbiota, bile flow and diet itself on EEC function.

The consensus view is that postprandial GLP-1 levels are slightly reduced in subjects with obesity (Adam and Westerterp-Plantenga 2005; Carr et al. 2010; Chia et al. 2017; Meyer-Gerspach et al. 2014; Verdich et al. 2001b) or prediabetes (Faerch et al. 2015), although this effect has not been found in all studies (Feinle et al. 2002; Seimon et al. 2013). This lowered GLP-1 secretion may be related to the impaired bile acid responses seen in obesity (Glicksman et al. 2010). Several studies have also identified reductions in fasting or postprandial $PYY_{3–36}$ (Batterham et al. 2003; Meyer-Gerspach et al. 2014) and CCK (Baranowska et al. 2000) levels in individuals with obesity, but data are inconsistent and the overall effect of high BMI remains controversial [reviewed in Steinert et al. (2017)]. There are some reports of increased GIP secretion in obese versus healthy-weight participants (Chia et al. 2017; Creutzfeldt et al. 1978), but others measure no change (Carr et al. 2010; Verdich et al. 2001b).

7.1 Alteration of EEC Expression Profiles by Diet or Obesity

Several groups have demonstrated that high-fat diet (HFD) feeding in animal models leads to altered EEC function (Dusaulcy et al. 2016; Richards et al. 2016; Ye et al. 2019). It appears that most effects are a result of the diet itself, rather than the resultant obesity, as they are not observed in genetically-obese leptin deficient mice (Aranias et al. 2015). These studies have not produced conclusive results, likely because any effect size is small, the macronutrient composition of HFD used is not standardised and studies of EEC cell number are typically reliant on inconsistent immunohistochemistry. PYY expression tends to increase (Larraufie et al. 2018; Richards et al. 2016), but was unchanged in one study (Aranias et al. 2015). The effect of HFD on *Gcg* expression is particularly controversial, with studies variably reporting an increase (Ahlkvist et al. 2013; Aranias et al. 2015), decrease (Richards et al. 2016), or opposite effects on L-cell number and circulating GLP-1 levels (Gniuli et al. 2010; Kappe et al. 2014). The most convincing mechanism underlying diet-induced alteration in hormone levels in the colon involves short-chain fatty acids (SCFAs), such as butyrate and propionate generated by microbial fermentation, modulating gene expression via L-cell FFA2/3 (Tolhurst et al. 2012; Zhou et al. 2008). There is a particularly strong effect of butyrate on *PYY* expression in human L-cells attributable to histone deacetylase (HDAC) activation (Larraufie et al. 2018). Small intestinal GIP expression following HFD has also been reported to increase (Gniuli et al. 2010) or decrease (Richards et al. 2016).

Despite some small changes in expression of nutrient-sensing receptors in mouse EECs following high-fat feeding (Peiris et al. 2018; Richards et al. 2016), there are no major alterations in expression of these receptors in lean and overweight humans (Baumard et al. 2021). It has also been suggested that there are no major differences in the transcriptome of jejunal EECs from lean and obese human subjects, although

this study was not sufficiently powered to detect subtle changes (Roberts et al. 2019). By contrast, a larger cohort of individuals with obesity identified small reductions in expression of transcription factors driving jejunal EEC differentiation compared to non-obese subjects, and co-morbidity with T2DM was associated with further altered gene expression and reduced jejunal GLP-1 levels (Osinski et al. 2021). While one clinical study reported an increased jejunal L-cell density people with obesity who self-reported consumption of a fat-rich diet (Aranias et al. 2015), there was no difference in colonic *GCG*, *PYY*, *CCK* or *SST* expression between lean and overweight individuals (Baumard et al. 2021).

Bariatric surgery drastically alters the exposure of EECs from different regions of the GI tract to incoming nutrients and therefore provides a particularly interesting model to assess the regulation of EEC function by diet. Despite these dramatic changes, it has been demonstrated in both humans and rodents that neither gastric bypass nor gastrectomy induces major differences in EEC density or transcriptome (Larraufie et al. 2019; Mumphrey et al. 2013; Rhee et al. 2015).

7.2 Maintained Gut Hormone Function in Obesity

In considering the therapeutic activation of anorexigenic gut hormone pathways, it is important to consider whether obesity is linked to hormone resistance, as is seen for insulin and leptin (Konner and Bruning 2012). In line with the success of GLP1R agonists, a meta-analysis demonstrated a similar effectiveness of active GLP-1 infusion in reducing food intake in obese and healthy-weight individuals (Verdich et al. 2001a). The satiety-inducing effects of $PYY_{3–36}$ are also maintained in obese subjects (Batterham et al. 2003). By contrast, there is evidence in rodents that HFD leads to loss of CCK's anorexigenic effects, possibly via leptin resistance in vagal afferent neurons (de Lartigue et al. 2012; Duca et al. 2013). Excessive GIP signalling in response to overnutrition, on the other hand, has been implicated in the development of leptin resistance at the level of the hypothalamus (Kaneko et al. 2019). The insulin-stimulating incretin effects of GLP-1 are maintained in T2DM, but those of GIP are impaired (Nauck et al. 1993); however, this appears linked to chronic hyperglycaemia, rather than obesity (Hojberg et al. 2009).

8 Conclusion

Gut hormones such as GLP-1, $PYY_{3–36}$, CCK and GIP released postprandially from EECs exert several actions which could be beneficial in the treatment of obesity: slowing of nutrient transit through the GI tract; stimulation of pancreatic insulin secretion to prepare other organs for an impending increase in circulating glucose levels; and direct signalling to the central nervous system to enhance satiation after a meal or maintain longer term satiety. The mechanisms of this central signalling remain under active investigation but regardless of how and to what extent gut-derived peptides modulate appetite under normal physiological conditions, gut

hormone receptors can be successfully targeted with peripherally-delivered drugs to pharmacologically reduce food intake in individuals with obesity.

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Insights from Studies of White Adipose Tissue Using Single-Cell Approaches

Niklas Mejhert and Mikael Rydén

Contents

Abstract

Technologies allowing studies at single-cell resolution have provided important insights into how different cell populations contribute to tissue function. Application of these methods to white adipose tissue (WAT) has revealed how various metabolic aspects of this organ, such as insulin response, inflammation and tissue expansion, are regulated by specific WAT resident cells, including different subtypes of adipocytes, adipocyte progenitors as well as immune and endothelial cells. In this chapter, we provide an overview of the different technical approaches, their strengths and weaknesses, and summarize how these studies have improved our understanding of WAT function in health and disease.

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Obesity · Single-cell sequencing · Single-nucleus sequencing · Spatial transcriptomics · Type 2 diabetes

1 Introduction

Adipose tissue can be anatomically and functionally subdivided into white and brown depots. Brown adipose tissue, the main function of which is to generate heat by oxidizing fatty acids and glucose, will not be covered in this section although there are publications at the single-cell level generated from murine models (Shamsi et al. 2021; Karlina et al. 2021; Song et al. 2020; Spaethling et al. 2016). However, while adult humans have cold-activated "beige" adipose tissue, they lack classical brown depots comparable to those in rodents and we will therefore not discuss these findings further herein. Instead, we will focus on results obtained by applying single-cell transcriptomic approaches to white adipose tissue (WAT) and briefly mention recent results in smaller depots such as perivascular and dermal adipose tissues.

From being regarded as a mere energy storing tissue, research in the last three decades has clearly identified WAT as a central metabolic organ, which impacts on multiple aspects of metabolism via both direct and indirect mechanisms (Rosen and Spiegelman 2014). There are several WAT depots, which are broadly subdivided into subcutaneous (e.g., abdominal, femoral, gluteal, dermal) or visceral (e.g., omental, mesenteric, epiploic, perivascular) depending on their anatomical location. Regardless of site, WAT is a loose connective tissue composed of many cell types including mature adipocytes, adipocyte progenitors, leukocytes, and vascular and smooth muscle cells. More recently, it has become clear that the interaction between different cell types resident in WAT is important in health and disease. For example, a healthy expansion of WAT mass is characterized by a coordinated remodeling of the cell composition, including increased adipocyte size and number, neovascularization (angiogenesis), and infiltration of specific immune cell populations. In contrast, an unhealthy WAT phenotype is linked to adipocyte hypertrophy, attenuated angiogenesis, and an increase in leukocytes with a more pro-inflammatory profile. This results in a chronic low-grade inflammation, hypoxia, and fibrosis which impact negatively on the lipid storage capacity of fat cells (Sun et al. 2011). Together, disturbances in these processes result in complications such as insulin resistance, type 2 diabetes, dyslipidemia, and cardiovascular disease (Scherer 2019; James et al. 2021). This has prompted an intense mapping and characterization of the cell types in WAT and how they are altered by obesity and other common metabolic disorders.

A prerequisite for studies of cellular composition with high granularity has been the development and application of next generation sequencing-based methods (Anaparthy et al. 2019), which have allowed analyses of adipose tissue composition at the single-cell level. These efforts have been excellently summarized in several recent reviews (Corvera 2021; Ferrero et al. 2020; Deutsch et al. 2020; Rondini and

Table 1 Summary of studies. The present chapter summarizes findings from several studies employing single-cell and single-nuclei approaches in different WAT depots. Due to space constraints, only a few of these studies have been discussed in more detail. Also, the table aims to give an overview of recent important contributions to the field, but it is not intended to cover all studies in adipose tissue biology employing single-cell approaches

First author	PMID	Year	Journal	Technique	Species and depot
Min, S.Y.	31420514	2019	Proc Natl Acad Sci U S A	Bulk RNA sequencing of single-cell colonies	Human (subcutaneous WAT from carotid endarterectomies)
Acosta, J.R.	29116032	2017	Stem Cell Res Ther	Single-cell transcriptomics	Human (abdominal subcutaneous WAT)
Tabula Muris Consortium	30283141	2018	Nature	Single-cell transcriptomics	Mice (inguinal, gonadal adipose, mesenteric WAT, and interscapular BAT)
Hepler, C.	30265241	2018	eLife	Single-cell transcriptomics	Mice (gonadal WAT)
Burl, R.B.	29937373	2018	Cell Metab	Single-cell transcriptomics	Mice (inguinal and epididymal WAT)
Schwalie, P.C.	29925944	2018	Nature	Single-cell transcriptomics	Mice (subcutaneous WAT)
Pan, X.X.	31087498	2019	Aging Cell	Single-cell transcriptomics	Mice (perivascular WAT from the thoracic aorta)
Zhang, Z.	31503545	2019	J Clin Invest	Single-cell transcriptomics	Mice (dermal fat)
Cho, D.S.	31767614	2019	Life Sci Alliance	Single-cell transcriptomics	Mice (epididymal WAT)
Weinstock, A.	31396408	2019	Immunometabolism	Single-cell transcriptomics	Mice (perigonadal WAT)
Gu, W.	31340667	2019	Arterioscler Thromb Vasc Biol	Single-cell transcriptomics	Mice (periaortic WAT surrounding thoracic aorta and cultured PV-ADSCs)
Jaitin, D.A.	31257031	2019	Cell	Single-cell transcriptomics	Mice (epididymal WAT) and human (omental WAT)
Spallanzani, R.G.	31053654	2019	Sci Immunol	Single-cell transcriptomics	Mice (epididymal WAT and additional tissues)
Merrick, D.	31023895	2019	Science	Single-cell transcriptomics	Mice (inguinal WAT) and human (abdominal/ flanking subcutaneous WAT)
Shook, B.A.	32302523	2020	Cell Stem Cell	Single-cell transcriptomics	Mice (dermal fat)
Ramirez, A.K.	32355218	2020	Nat Commun	Single-cell transcriptomics	Human (abdominal subcutaneous WAT)
Henriques, F.	32755590	2020	Cell Rep	Single-cell transcriptomics	Mice (inguinal WAT)
Oguri, Y.	32615086	2020	Cell	Single-cell transcriptomics	Mice (BAT and inguinal as well as epididymal WAT)
Vijay, J.	32066997	2020	Nat Metab	Single-cell transcriptomics	Human (subcutaneous and visceral WAT)
Hildreth, A.D.	33907320	2021	Nat Immunol	Single-cell transcriptomics	Human (deep subcutaneous WAT from abdominoplasty)

(continued)

Table 1 (continued)

First author	PMID	Year	Journal	Technique	Species and depot
Suwandhi, L.	33707431	2021	Nat Commun	Single-cell transcriptomics	Mice (subcutaneous WAT)
Rajbhandari, P.	31644425	2019	eLife	Single-nucleus and single-cell transcriptomics	Mice (inguinal WAT)
Angueira, A.R.	33846639	2021	Nat Metab	Single-nucleus and single-cell transcriptomics	Human and mouse (periaortic WAT)
Sun, W.	33116305	2020	Nature	Single-nucleus RNA sequencing	Mice (interscapular BAT) and human (deep-neck BAT and subcutaneous WAT)
Sárvári, A.K.	33378646	2021	Cell Metab	Single-nucleus RNA sequencing	Mice (epididymal WAT)
Bäckdahl, J.	34380013	2021	Cell Metabolism	Spatial transcriptomics	Human (abdominal subcutaneous WAT)

Granneman 2020). Despite the fact this field of research has been established only recently, a number of important studies have contributed to our understanding of WAT cell heterogeneity and function. In this chapter, we will discuss the major insights from these studies with a particular focus on subcutaneous and visceral WAT and key results from selected studies are presented below and summarized in Table 1.

Provided that this book focuses on the metabolic role of adipose tissue, we will not cover in detail single-cell studies performed on smaller WAT depots such as those in proximity to large vessels (perivascular adipose tissue) (Pan et al. 2019; Gu et al. 2019; Angueira et al. 2021) or within the skin (dermal adipose tissue) (Zhang et al. 2019; Shook et al. 2020). Nevertheless, these studies have revealed that perivascular and dermal depots contain multiple cell populations. The pathophysiological role of the cellular heterogeneity identified by single-cell data has been demonstrated by follow-up experiments based on imaging, lipidomic and genetic experiments in mice. Thus, in perivascular depots, the adipocyte progenitors that differentiate into thermogenic (beige) adipocytes have been identified (Angueira et al. 2021), while in dermal tissue, specific populations of adipocytes have been shown to regulate wound healing (Zhang et al. 2019; Shook et al. 2020). However, as the bulk of these studies have been performed in murine models, the clinical relevance of these findings remains to be tested.

2 Single-Cell Technologies: A Rapidly Evolving Field

Single-cell sequencing can be applied to different starting materials, i.e. DNA and RNA. Analyses of DNA (e.g., genomic sequencing and cytosine-methylation as well as chromatin accessibility assessments) are particularly relevant in cancer and microbiota research, where mutations in genomic DNA and/or epigenetic (dys)-regulations are important drivers in carcinogenesis and virulence, respectively. In this chapter we will focus on analyses of gene expression as this is the primary aspect studied in WAT biology. Sequencing of single cells can be performed with a variety of technical platforms, including 10x Chromium, Smart-seq2, and CEL-seq2, just to name a few. All have different strengths and weaknesses, and because the technical innovations and the bioinformatic pipelines in this area are developing very fast, we refer the reader to recent literature for more detailed explanations of the techniques and the analyses performed (Andrews et al. 2021; Ding et al. 2020; Slyper et al. 2020). Importantly, while there are a few direct head-to-head comparisons in some cell types (Ding et al. 2020; Natarajan et al. 2019), there have so far been no efforts to assess the different approaches in WAT. Therefore, there is currently no gold standard in the field of WAT research.

3 Platforms to Study White Adipose Tissue Heterogeneity

A major challenge in studying WAT is that adipocytes, which constitute over 90% of the tissue volume, are inherently difficult to work with ex vivo as they are large, buoyant, and fragile. Therefore, standard approaches to obtain single cells, including magnetic and fluorescence-activated cell sorting (FACS) or microfluidics, cannot be used. As a consequence, efforts have been made to develop instruments that allow sorting of mature adipocytes, but their use has so far been limited (Wang et al. 2020; Hagberg et al. 2018). On the other hand, the stromal vascular fraction (SVF) of WAT, which contains the non-adipocyte cells (constituting >60–80% of the total cell number in the tissue), is well-suited for standard single-cell preparations. Therefore, the majority of the studies on WAT have been performed on unsorted SVF or purified cell populations from the SVF using fluorescent markers. Common markers for sorting SVF and obtaining specific cellular subsets are, e.g., CD45, CD31, CD34, Lin, and Sca-1 but also several others as extensively overviewed by Cawthorn et al. (Cawthorn et al. 2012).

A drawback with single-cell analyses is that in addition to the sorting procedure, the tissue needs to be dissociated using rather harsh protocols, e.g. enzymatic degradation at 37°C, which may influence downstream results (Denisenko et al. 2020). An alternative approach, snRNA-seq, that has gained much attention in recent years is therefore to isolate single-nuclei from tissue pieces and use these to generate transcriptional profiles (for a WAT-based protocol, see Van Hauwaert et al. (2021)). An advantage with this method is that it generates data from both stromavascular cells and mature adipocytes from the same sample (Miao et al. 2020; Jew et al. 2020; Karunakaran et al. 2020; Alvarez et al. 2020; Sarvari et al. 2021; Sun et al. 2020), but a caveat is that the analyses are performed on nuclear RNA, which may differ from the profiles of whole cell RNA (Ding et al. 2020; Denisenko et al. 2020). Complementing these two approaches is spatial transcriptomics, which comprises a range of technical platforms, that allows transcriptomic analyses in histological sections (Rao et al. 2021). An obvious advantage with these spatial mapping methods is that they provide information on the tissue microarchitecture and the localization of specific cell types in situ. The high-throughput data generated from these different approaches have prompted bioinformatic developments that allow integration of scRNA-seq/snRNA-seq and spatial transcriptomic data (Moncada et al. 2020; Andersson et al. 2020).

4 Exposures of Interest in Single-Cell Studies of White Adipose Tissue

Single-cell analyses in WAT have mainly focused on comparisons of lean and obese humans and/or mice fed chow or high fat diets. As discussed more in detail below, these efforts have provided insights into how changes in WAT amount affects the total number and proportion of different cells as well as their phenotypes. In addition to this, some groups have been interested in WAT beigeing/browning, a process

where specific white adipocytes become more thermogenic upon stressors such as cold exposure.

5 Results Related to Specific White Adipose Tissue Resident Cells

5.1 Adipocyte Progenitors

Data based on ^{14}C dating of genomic DNA have shown that mature fat cells are turned over at a 10% annual rate in adult humans (Spalding et al. 2008). This implies that fat cells are continuously generated and suggests that adipocyte progenitors (APs) present in WAT (or from the circulation (Ryden et al. 2015)) are required for fat cell formation. While adipogenesis has been extensively studied in vitro, much less is known about APs in vivo. Earlier work by several investigators using FACS as well as genetic and transplantation models in mice identified AP populations with distinct cell surface markers, tissue localization, and differentiation capacities (Berry et al. 2014; Jeffery et al. 2015, 2016; Gupta et al. 2012; Shao et al. 2018; Gupta et al. 2010; Berry and Rodeheffer 2013; Rodeheffer et al. 2008). However, an inherent weakness with these approaches was that the analyses were to a large degree adopting bulk analyses of specific cell surface markers. These limitations have been resolved by transcriptional studies at the single-cell level. Some of the key outstanding questions in the field that are now possible to address are the following:

- What defines APs in terms of gene expression profiles?
- Are there different AP subtypes, and if so, what are their characteristics?
- How do specific AP subtypes contribute to WAT function?

An early study based on APs isolated by FACS from human subcutaneous WAT suggested that these cells constitute a homogenous population (Acosta et al. 2017). This notion was subsequently challenged by larger studies in both human (Sun et al. 2020; Merrick et al. 2019; Vijay et al. 2020) and murine WAT (Sarvari et al. 2021; Merrick et al. 2019; Hepler et al. 2018; Burl et al. 2018; Schwalie et al. 2018; Cho et al. 2019; Suwandhi et al. 2021; Tabula Muris Consortium et al. 2018; Rajbhandari et al. 2019), demonstrating that both the subcutaneous and visceral depots contain multiple subtypes of APs. More specifically, several of these reports have identified a hierarchy among APs that seems to reflect different degrees of adipocyte lineage commitment. Thus, one cell class is marked by DPP4/Pi16/CD55 expression and has been shown to be highly proliferative (Sarvari et al. 2021; Merrick et al. 2019; Burl et al. 2018; Schwalie et al. 2018; Suwandhi et al. 2021). These multipotent APs are localized to the reticular interstitium (Merrick et al. 2019) and are suggested to constitute the primordial cell which then gives rise to more committed progenitors. The latter are termed preadipocytes and are characterized by multiple markers, including ICAM/CD54 as well as the expression of genes linked to later stages of

adipogenic differentiation (Sarvari et al. 2021; Hepler et al. 2018; Burl et al. 2018; Schwalie et al. 2018).

In addition to these two cell classes, where there is a consensus between studies, at least two anti-adipogenic AP subtypes have been identified; Schwalie et al. isolated *CD142*/*Clec11a*-expressing "Aregs" from murine subcutaneous WAT which were shown to be anti-adipogenic both in vitro and in vivo (Schwalie et al. 2018), while Hepler and colleagues found "Fibro-inflammatory progenitors", an $LY6C^+/PDGFR\beta^+/DPP4^+$ subtype in murine visceral WAT, with similar anti-adipogenic properties (Hepler et al. 2018). In subsequent studies of murine WAT, $CD142^+/Clec11a^+$ APs were confirmed to be enriched in visceral vs. subcutaneous WAT, and the proportion was higher in the obese compared with the lean state (Merrick et al. 2019). However, in contrast to Schwalie et al., these cells were shown to be pro-adipogenic (Merrick et al. 2019), indicating that the role of this cell type is unclear. Underlying these differences could be multiple technical aspects including the usage of different FACS strategies to isolate the SVF.

Several investigators have aimed to identify APs with the propensity to form beige, thermogenic adipocytes (Hepler et al. 2018; Suwandhi et al. 2021; Oguri et al. 2020). Significant findings from these efforts were the identification of $CD81^+$ (Oguri et al. 2020) and $Asc1^-$ (Suwandhi et al. 2021) subpopulations of cells in murine WAT that can form beige adipocytes upon classical "browning" cues such as cold exposure or beta-adrenergic stimulation. Moreover, depletion of these cells impacts on whole body energy metabolism in murine gene knockout models, suggesting a potential functional role of these APs, at least in mice. Altogether, these analyses provide evidence for multiple APs with specific functional characteristics in murine and human WAT. Although there is a reasonable consensus regarding the findings in rodents, the picture is much less clear in human WAT including the difference in AP subtypes between WAT depots (Vijay et al. 2020). Provided that there are now multiple studies using single-cell sequencing in human WAT, a meta-analysis of the data would help to provide a common ground on human AP heterogeneity. Moreover, to what degree these APs impact on human WAT function remains to be shown.

5.2 Immune Cells

The fact that circulating immune cells have been extensively mapped for different cell surface markers has facilitated the isolation and characterization of different cell populations resident in WAT using FACS. However, more recent single-cell-based studies have not only confirmed FACS-based findings but have also provided further insights into this field. Specifically, both subcutaneous and visceral WAT depots have been studied in humans and mice comparing lean and obese states (Rajbhandari et al. 2019; Jaitin et al. 2019; Hildreth et al. 2021; Spallanzani et al. 2019; Weinstock et al. 2019). Among other things, this has revealed new subpopulations of immune cells present only under specific conditions. For example, Jaitin et al. identified lipid-associated macrophages (LAMs), which were characterized by *Trem2* expression

and constituted the most significantly up-regulated macrophage population in mice fed a high fat diet (Jaitin et al. 2019). The presence of LAMs and their enrichment in the obese state was subsequently confirmed in human WAT (Hildreth et al. 2021). In mice, genetic labeling studies and pseudotime analyses revealed that these cells derive from circulating monocytes and infiltrate WAT upon tissue expansion. LAMs exhibit phagocytic properties and seem to be important in protecting against the lipotoxic effects of fat storage. In line with this, *Trem2* knockout mice displayed a depletion of LAMs in WAT, increased fat mass, adipocyte hypertrophy, dyslipidemia, and glucose intolerance. These results support previous studies (Wernstedt Asterholm et al. 2014), demonstrating that immune cell infiltration into WAT can have beneficial effects to enable a healthy tissue remodeling.

5.3 Mature Adipocytes

As discussed above, the inherent difficulties in sorting mature fat cells have so far limited high-throughput analyses of this population by single-cell isolation (Wang et al. 2020; Hagberg et al. 2018). Instead, snRNA-seq has been used in both murine and human WAT to help identify fat cell heterogeneity. This has enabled the identification of mature white adipocyte subtypes that display a thermogenic capacity, which may facilitate WAT beigeing upon cold exposure (Sun et al. 2020; Rajbhandari et al. 2019). The presence of such cells was confirmed in human WAT (Sun et al. 2020), although the pathophysiological role of these cells in man is less clear. In additional efforts, Sarvari et al. described the presence of mature fat cell types in murine epigonadal WAT, with distinct transcriptional profiles including "lipogenic" and "stressed lipid-scavenging" subtypes where high fat diet decreased the proportion of the former and increased that of the latter (Sarvari et al. 2021). While these studies suggest that also adipocytes constitute a heterogeneous population, they were not able to identify the localization of these cells in the tissue or their relationship to other cells, including APs.

The limitations described above have prompted several groups to use other methods to study single mature fat cells. Min et al. obtained APs from ex vivo cultured human WAT explants and generated monoclonal cell populations that were further characterized (Min et al. 2019). Following adipogenic induction in vitro, individual cell clones gave rise to four different subtypes of differentiated adipocytes, out of which one was characterized as "beige" and displayed a pronounced response to the adenylyl cyclase-activator forskolin. Among the white adipocytes, the most prominent feature was the differential expression of leptin and adiponectin in two of the subtypes. While there was some gene expression overlap between the two populations, the adiponectin-enriched cells expressed many of the classical lipogenic genes found in mature white fat cells. These findings were corroborated using the Visium spatial transcriptomics platform, which provides a resolution of 55 μm (Backdahl et al. 2021). Given the relatively large size of adipocytes, this is close to the single-cell level. Based on analyses of human WAT, spatial mapping allowed the identification of 18 different cell types, which

could be broadly categorized into APs, mature adipocytes as well as immune and vascular cells. Within the mature adipocyte class, three distinct subtypes were found; each enriched for a specific set of genes, including the well-established adipokines leptin, adiponectin, and retinol-binding protein-4. Further analyses based on samples from subjects before and after a hyperinsulinemic euglycemic clamp revealed that the transcriptional response to insulin was confined to adipocytes enriched for adiponectin. Taken together, these high-resolution RNA mapping approaches demonstrate that WAT is composed of different fat cell populations with distinct responses to external cues such as forskolin and insulin. The next steps will be to generate and/or isolate these subtypes and characterize them further.

6 Future Perspectives

The application of single-cell analyses to study WAT has progressed very fast and has allowed the identification of different cell types present in multiple WAT depots. While effects of high fat diet/obesity and beigeing have been reported, most of the results pertain to murine WAT. Moreover, a limitation in comparing studies is that they have used different sequencing platforms, cell isolation protocols, WAT depots, murine strains, and interventions. What is currently lacking in this field are follow-up studies interrogating the function of the different subpopulations both in vitro and in vivo (Kim et al. 2021; Tanay and Regev 2017). It would also be interesting to apply these technologies to prospective intervention studies focusing on the effects of specific medications, nutrients/diets, and other external cues on WAT. Results from such studies could help us link clinical outcomes to specific cell types and thereby enable the development of more targeted treatments in the era of precision medicine.

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Obesity-Related Insulin Resistance: The Central Role of Adipose Tissue Dysfunction

Gabriele Mocciaro and Amalia Gastaldelli

Contents

Abstract

Obesity is a key player in the onset and progression of insulin resistance (IR), a state by which insulin-sensitive cells fail to adequately respond to insulin action. IR is a reversible condition, but if untreated leads to type 2 diabetes alongside increasing cardiovascular risk. The link between obesity and IR has been widely investigated; however, some aspects are still not fully characterized.

In this chapter, we introduce key aspects of the pathophysiology of IR and its intimate connection with obesity. Specifically, we focus on the role of adipose tissue dysfunction (quantity, quality, and distribution) as a driver of whole-body IR. Furthermore, we discuss the obesity-related lipidomic remodeling occurring in adipose tissue, liver, and skeletal muscle. Key mechanisms linking lipotoxicity to IR in different tissues and metabolic alterations (i.e., fatty liver and diabetes)

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and the effect of weight loss on IR are also reported while highlighting knowledge gaps.

Keywords

Adipose tissue insulin resistance · Ceramides · Diacylglycerols · Hepatic insulin resistance · Lipidomic remodeling · Lipotoxicity · Muscle insulin resistance

1 Introduction

Insulin resistance (IR) is very common in subjects with obesity and is related to the impairment of the insulin action on glucose, fatty acid, and amino acid metabolism. For this reason, all organs manifest signs of IR.

There are several ways to assess in vivo IR. The gold standard is the euglycemic-hyperinsulinemic clamp developed by DeFronzo et al. (1979). This technique requires the injection of insulin alongside glucose so that glycemia is kept stable. In healthy subjects, the elevated insulin levels almost entirely suppress endogenous glucose production (EGP) so the rate of exogenous glucose infusion provides an estimate of peripheral glucose uptake that is an index of muscle insulin sensitivity. Because of the invasiveness and technical skills required to perform the clamps, several surrogate markers have been developed. A summary of the most common indices to assess IR and insulin sensitivity is reported in Table 1.

The metabolism of the adipose tissue (AT) is crucial to understand how obesity is linked to IR. In obese subjects, most of the defects in insulin action are caused by lipotoxicity that is due to the synthesis and accumulation of lipotoxic species within the cells. These defects are often reversible as demonstrated by major weight loss obtained by either very low caloric diet (as demonstrated by the DiRECT trial (Lean et al. 2018, 2019)) or by bariatric surgery (Camastra et al. 2011; Greco et al. 2002; Stenberg and Thorell 2020).

The composition of AT and circulating lipids can be evaluated using lipidomic techniques, based on chromatography coupled with mass spectrometry (MS) or nuclear magnetic resonance (NMR) spectroscopy, that allow the extensive measurement of lipid composition of cells, tissues, and biological fluids (Liggi and Griffin 2017). The field has greatly benefited from the advances in mass-spectrometry technologies, which allow the measurements of hundreds of lipids in few minutes (~3 min) (Liggi and Griffin 2017). A description of MS and NMR technologies is beyond the scope of this chapter but a thorough review can be found elsewhere (Griffin et al. 2011).

As a consequence of IR, obese subjects have excessive lipolysis and lipogenesis, dysregulation of glucose metabolism, which leads to the development of glucose intolerance and type 2 diabetes (T2D), and of amino acid metabolism, characterized by enhanced catabolism and diminished anabolism, ultimately leading to sarcopenic obesity (Linge et al. 2020). In obese subjects, the increased fat mass (up to 50%)

Table 1 Summary of the most common indices for insulin resistance/insulin sensitivity

Indices	Metabolic state	Condition	Tissue	Formula	Reference
Adipo-IR	Fasting	Resistance	Adipose	FFA * I_0 mU/l	(Groop et al. 1989; Gastaldelli et al. 2017)
ATIRI	Fasting	Resistance	Adipose	RaPalmitate * I_0 mU/l	(Fabbrini et al. 2012)
Lipo-IR	Fasting	Resistance	Adipose	RaGly * I_0 mU/l	(Rosso et al. 2019)
Belfiore ISI_{FFA}	OGTT	Sensitivity	Adipose	2/[(AUC-I * AUC-FFA) + 1]	(Belfiore et al. 1998)
Hep-IR	Fasting	Resistance	Liver	EGP * I_0 mU/l	(Groop et al. 1989; Gastaldelli et al. 2007; Abdul-Ghani et al. 2007)
HepIR OGTT	OGTT	Resistance	Liver	(G_0 mg/dl + G_{30} mg/dl) / 100 / 2 * (I_0 mU/ml + I_{30} mU/ml) / 2	(Abdul-Ghani et al. 2007)
LIRI	OGTT	Resistance	Liver	- 0.091 + LOG (I mean * 6) * 0.4 + LOG (FM / weight * 100) * 0.346 – LOG HDL-C mg/dl * 0.408 + LOG BMI * 0.435	(Vangipurapu et al. 2011)
TG/HDL-Chol	Fasting	Resistance	Liver	Tg/HDL-Chol	(Kim-Dorner et al. 2010)
Hep-IR	Fasting	Resistance	Liver	EGP * I_0 mU/l	(Groop et al. 1989)
HOMA-IR	Fasting	Resistance	Peripheral	(I_0 mU/ml * G_0 mmol/l)/ 22.5	(Matthews et al. 1985)
QUICKI	Fasting	Sensitivity	Peripheral	1/(LOG I_0 mU/ml + LOG G_0 mg/dl)	(Katz et al. 2000)
FIRI	Fasting	Resistance	Peripheral	(I_0 mU/ml * G_0 mg/dl) / 25	(Duncan et al. 1995)
IGR	Fasting	Resistance	Peripheral	I_0 mU/ml / G_0 mg/dl	(Hanson et al. 2000)
ISI Bennett	Fasting	Sensitivity	Peripheral	1/(ln G_0 mg/dl * ln I_0 mU/l)	(Anderson et al. 1995)
Belfiore ISI_{gly}	OGTT or meal	Sensitivity	Peripheral	2/[(AUC-I * AUC-G) + 1]	(Belfiore et al. 1998)
OGIS	OGTT or meal	Sensitivity	Peripheral	f (G_0, G_{90}, G_{120}, I_0, I_{90}, D)[a]	(Mari et al. 2001)

(continued)

Table 1 (continued)

Indices	Metabolic state	Condition	Tissue	Formula	Reference
ISI Matsuda	OGTT or meal	Sensitivity	Peripheral	$10^4/\sqrt{}$ [(G_0 mg/dl * I_0 mU/ml) * (G_{mean} * I_{mean})]	(Matsuda and DeFronzo 1999)
SiOGTT	OGTT	Sensitivity	Peripheral	1/[LOG(G_0 + G_{30} + G_{90} + G_{120}) mg/dl + LOG (I_0 + I_{30} + I_{90} + I_{120}) mU/ml]	(Bastard et al. 2007)
BIGTT	OGTT	Sensitivity	Peripheral	EXP (4.9-(0.00402 * I_0 pmol/l) – (0.000556 * I_{30} pmol/l) – (0.00127 * I_{90} pmol/l) – (0.152 * G_0 mmol/l) – (0.00871 * G_{30} mmol/l) – (0.0373 * G_{120} mmol/l) – (0.145 * gender) – (0.0376 * BMI)	(Hansen et al. 2007)
ISI Stumvoll Dem	OGTT	Sensitivity	Peripheral	0.226–0.0032 * BMI -0.0000645 * I_{120}(pmol/l) - 0.000375 * I_0(pmol/l) - 0.00519 * G_{90} (mmol/l)	(Stumvoll et al. 2000)
$eMCR^{dem}$	OGTT	Sensitivity	Peripheral	18.8–0.271 * BMI – 0.0052 * I_{120} pmol/l – 0.27 * G_{90} mmol/l	(Stumvoll et al. 2000)
ISI Stumvoll Nodem	OGTT	Sensitivity	Peripheral	0.157–0.00004576 * I_{120}(pmol/l) - 0.000299 * I_0(pmol/l) - 0.00519 * G_{120}(mmol/l)	(Stumvoll et al. 2000)
$eMCR^{nodem}$	OGTT	Sensitivity	Peripheral	13–0.0042 * I_{120} pmol/l – 0.384 * G_{90} mmol/l – 0.0209 * I_0 pmol/l	(Stumvoll et al. 2000)

Note: *ALB* albumin, *ALT* alanine aminotransferase, *AST* aspartate aminotransferase, *AUC* area under the receiver operating curve, *BIGTT*, β-cell function, insulin sensitivity index derived from oral glucose tolerance test, *BMI* body mass index, $eMCR^{dem}$ metabolic clearance rate estimation including demographic parameters, $eMCR^{nodem}$ metabolic clearance rate estimation without demographic parameters, *FIRI* fasting insulin resistance index, *HDL-C* high density lipoprotein cholesterol, *G* glucose, *HepIR OGTT* hepatic insulin resistance index, *HOMA* homeostasis model of assessment, *I* insulin, *IFG* impaired fasting glucose, *IGR* insulin to glucose ratio, *IR* insulin resistance, *ISI* insulin sensitivity index, *LIRI* liver insulin resistance index, *NFS* nonalcoholic fatty liver disease fibrosis score, *OGIS* oral glucose insulin sensitivity index, *QUICKI* quantitative insulin sensitivity check index, *SiOGTT* insulin sensitivity index derived from oral glucose tolerance test, *TG* triglycerides

[a]G_0, G_{90} and G_{120} are the plasma concentration of glucose measured at baseline, 90 and 120 min during OGTT; I_0, and I_{90} are the plasma concentration of insulin measured at baseline and 90 min during OGTT. D is the oral glucose dose (g/m_2 body surface area). The formula can be found at the following website: http://webmet.pd.cnr.it/ogis/

(Davidson et al. 2018), and the consequential loss of lean body mass, is a major determinant of impaired metabolism.

In this chapter, we describe how and why IR develops in obese subjects, highlighting the importance of AT composition and distribution in this process.

2 Adipose Tissue Metabolism, Composition, and Insulin Resistance

AT metabolism is a balance between lipogenesis and lipolysis. Insulin is an adipogenic hormone that promotes triglyceride (TG) synthesis and the accumulation of fat as lipid droplets while inhibiting lipolysis. AT size and composition are increasingly recognized as important players in the development of IR.

2.1 Impact of Insulin on Lipogenesis and Adipose Tissue Expansion

AT is the main energy storage site of the body due to its capability to accumulate TGs in lipid droplets. In obese subjects the lipogenesis and accumulation of TGs in both visceral and subcutaneous AT is increased compared to lean (Dadson et al. 2017). The synthesis of TG requires glucose to synthesize glycerol-3P that is then used to form diacylglycerol (DAG), then diacylglycerol acyltransferase (DGAT) catalyzes the formation TG from DAG and Acyl-CoA (Fig. 1). DGAT activity is highly sensitive to insulin action and upregulated in obesity (Ranganathan et al. 2006).

Among the other genes involved in lipogenesis, fatty acid synthase (FAS), involved in the synthesis of palmitate, is also sensitive to insulin action and upregulated in obesity (Ranganathan et al. 2006). However, even if the enzymes required for de novo lipogenesis (DNL) have been found in the AT, DNL does not make a major contribution in this tissue (Guo et al. 2000).

Insulin plays a role also in white adipose tissue expansion (WAT). Subjects with severe obesity have an enormous fat mass that may contribute up to more than 50% of total body weight (Davidson et al. 2018) and this is very often associated with the severity of IR. This WAT expansion occurs through the enlargement of adipocytes (becoming hypertrophic) or the increase in adipocyte number (hyperplasia) (Spalding et al. 2008; Jo et al. 2009). Many researchers have described the association between AT size and insulin concentrations or IR leading to the hypothesis that until the AT expands to buffer the excess caloric intake its lipid metabolism and lipotoxicity are maintained under control (Arner et al. 2010; Yang et al. 2012; Kim et al. 2007; Medina-Gomez et al. 2007); as the WAT becomes dysfunctional excess lipids are stored as visceral fat and as ectopic fat (e.g. in liver, muscle and pancreas, Fig. 2). The phenotype of subjects with small subcutaneous adipocytes that could expand to store the excessive caloric intake was considered "favorable" and at low risk to develop IR (Arner et al. 2010; Azzu et al. 2020; Mileti et al. 2021). However,

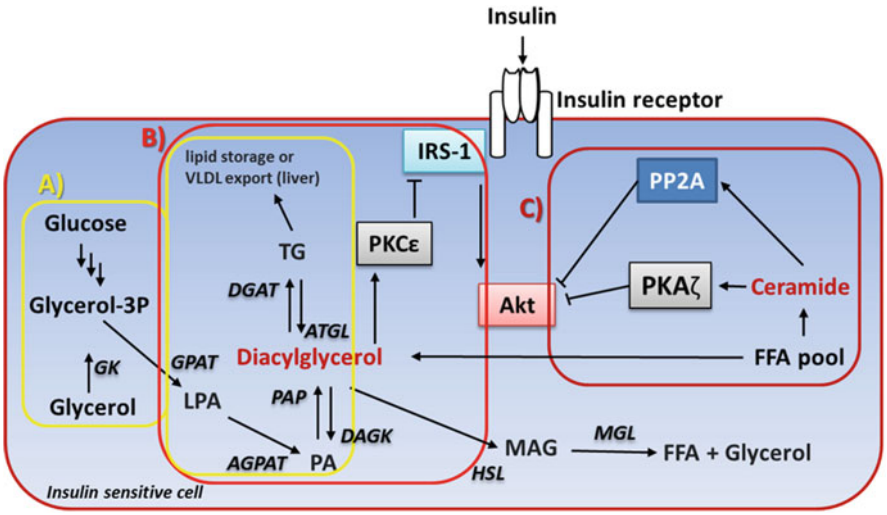

Fig. 1 Simplified figure of TG lipolysis and lipogenesis alongside lipotoxic mechanisms leading to IR in insulin-sensitive cells. (**A**) Glycerol-3P can derive from glucose (glycolysis) or glycerol due to the enzyme glycerol kinase (mostly liver). Once formed, Glycerol-3P can be converted into lysophosphatidic acid (LPA) by the enzyme glycerol-phosphate acyltransferase (GPAT). LPA is subsequently acylated by the enzyme acyl glycerol-phosphate acyltransferase (AGPAT), thus forming PA. The latter can then be dephosphorylated by phosphatidic acid phosphorylase (PAP) leading to the formation of diacylglycerol (DAG). The latter can be phosphorylated back to PA via the enzyme diacylglycerol kinase (DAGK) or further hydrolyzed into monoacylglycerol (MAG) by the enzyme hormone-sensitive lipase (HSL) and subsequently converted into FFA and glycerol by monoacylglycerol lipase (MGL). Alternatively, DAG can be acylated by diacylglycerol acyltransferase (DAGT) into triacylglycerol (TG) which are stored within the cell lipid pool or exported into the circulation as VLDL (only liver). Conversely, TG can be deacylated back to DAG by TG lipases, such as adipose triglyceride lipase (ATGL) or hormone-sensitive lipases (HSL). (**B**) DAG exert their lipotoxic effect through the activation of the protein kinase ε (PKCε) that binds to insulin receptor and inhibits its tyrosine kinase activity interfering with the ability of insulin to phosphorylate IRS-1, thus short-circuiting the signal propagation through the protein kinase B (Akt). (**C**) Ceramides promote IR via the inhibition Akt through the activation of two enzymes: (a) the protein phosphatase 2A (PP2A), and (b) PKA isoform zeta (ζ)

this has been proven to be not always the case. Johannsen and colleagues have shown that overfeeding resulting in significant weight gain (7 kg 55% of fat) was not associated to ectopic lipid accumulation in subjects with large adipocytes in subcutaneous WAT; in contrast, those with smaller fat cells displayed a worsened metabolic response to overfeeding with more severe IR (Johannsen et al. 2014). This is not against the theory of adipocyte expandability, but rather it suggests that only subjects that carry adipocytes that can adapt to excess energy by changing their size are indeed protected from obesity and ectopic fat accumulation. Indeed, Alligier et al. have shown that healthy lean subjects respond differently to overfeeding according to regulation of lipid storage-related genes (e.g., DGAT2, SREBP1c, and CIDEA) within the subcutaneous fat, i.e., those with a defective regulation of

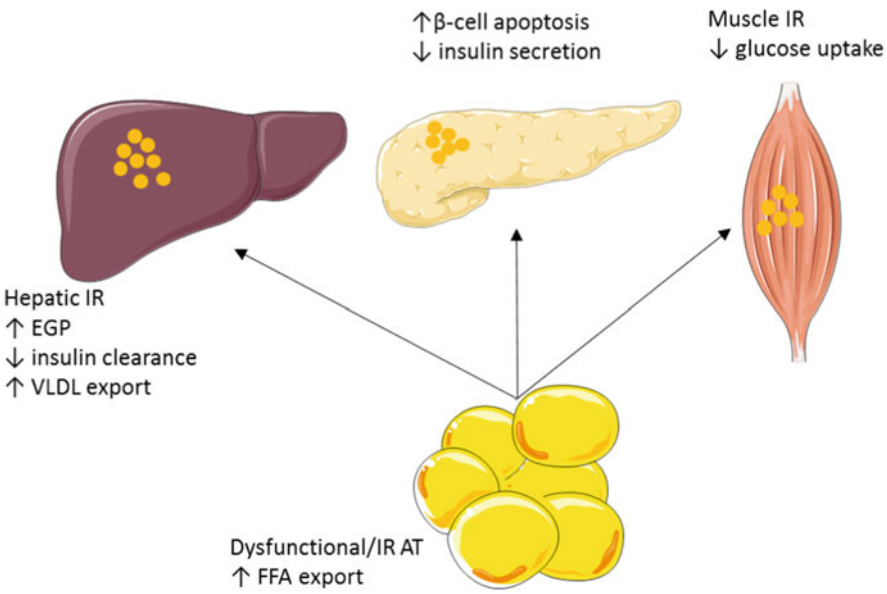

Fig. 2 Simplified representation of the deleterious effects of ectopic fat deposition following a dysfunctional and insulin resistant adipose tissue state. *IR* insulin resistance, *EGP* endogenous glucose production, *VLDL* very low-density lipoprotein, *AT* adipose tissue, *FFA* free fatty acids

these genes stored most of the excess calories in visceral rather than peripheral fat despite a similar increase in body weight (Alligier et al. 2013). Thus, subjects with subcutaneous adipocytes able to expand in response to excess energy intake are those at high risk to develop severe obesity while the others have higher risk of nonalcoholic fatty liver disease (NAFLD) and VAT accumulation with a "lipodystrophic" phenotype, i.e. with less subcutaneous fat but higher visceral and hepatic fat (Polyzos et al. 2019; Garg 2011).

2.2 Impact of Insulin on Lipolysis

Insulin exerts an inhibitory effect on AT lipolysis but in condition of IR there is an excess lipolysis leading to a chronic increase in serum nonesterified fatty acids (FFA) despite high serum insulin levels (Groop et al. 1989; Bonadonna et al. 1990; Gastaldelli et al. 2017; Boden et al. 1998; van Vliet et al. 2020; Sondergaard et al. 2017). This is evident both during an insulin-stimulated condition and fasting state (Bonadonna et al. 1990; Gastaldelli et al. 2009, 2017; Lomonaco et al. 2012; Bril et al. 2017; Bell et al. 2012; Brouwers et al. 2017). A lack of insulin action in the adipose tissue results in excess lipolysis. For this reason, most of the indexes that measure adipose tissue insulin resistance are based on the product of insulin and FFA or lipolysis (Table 1).

The majority of circulating FFA derive from hydrolysis of triglycerides (TG) in the subcutaneous adipose tissue (SAT) because a) SAT is the most important storage of TGs, b) TGs stored in the liver are released as lipoproteins and not as FFA, c) the amount of FFA that might derive from VAT is limited by the lower size of VAT compared to SAT, d) VAT releases FFA in the hepatic portal vein where they are probably all metabolized by the liver without appearing in the peripheral circulation.

Lipolysis involves 3 steps (Fig. 1), i.e., (1) the conversion of TGs into DAGs catalyzed by adipose TG lipase (ATGL or *Pnpla2*) (Zimmermann et al. 2004), (2) the conversion of DAG into MAG catalyzed by the hormone-sensitive lipase (HSL) (Haemmerle et al. 2002), (3) the hydrolysis of MAG into glycerol and FA catalyzed by monoacylglycerol lipase (MGL) (Fredrikson et al. 1986). ATGL plays a major role in systemic insulin sensitivity. Studies in mice with global or AT KO of ATGL (*Pnpla2*$^{-/-}$ mice) have demonstrated improved systemic insulin sensitivity despite impaired AT lipolysis, increased TG accumulation in AT, and reduced circulating plasma FFA and TG (Haemmerle et al. 2006; Trites and Clugston 2019).

Total AT lipolytic flux (umol/min) is enhanced in obesity and in IR, given the expansion of fat mass, while the capacity of FFA and glycerol release per unit of fat mass is reduced (Camastra et al. 2011; McQuaid et al. 2011; Mittendorfer et al. 2016). This evidence indicates that elevated circulating FFA levels are the consequence of excess WAT rather than an increased adipocyte lipolytic rate. In line with this study, by employing ^{14}C dating method to study long-term WAT lipid age, Arner et al. showed that obese people have indeed a reduced lipid turnover (Arner et al. 2011). It is worth mentioning that the study of AT turnover in health and disease has been hampered by the complexity of directly labeling pathway precursors in AT coupled with the slow AT turnover.

Not only excess caloric intake but also dietary composition might be implicated in the development of AT IR. Luukkonen et al. have shown that fasting lipolysis was increased after overfeeding with saturated fatty acids compared to similar overfeeding with unsaturated fat or carbohydrates (Luukkonen et al. 2018) due to higher IR of the AT.

2.3 Impact of Adipose Tissue Composition on IR

Not only general obesity but also fat composition and distribution have an important impact on glucose and lipid metabolism.

Although adipocytes contain mainly TGs, other lipids are stored in the adipose tissue and they might play a role in IR. Human lipidome includes thousands of distinct lipid molecular species that can be grouped into six major categories: fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, sterols, and prenols (Liebisch et al. 2020; Yang and Han 2016).

Among sphingolipids, ceramides are a crucial component of the cell membranes, second messengers, and have consistently been reported to be elevated in the WAT of subjects with IR and obesity as compared to healthy controls. Specifically, Kolak et al. showed that the SAT of IR subjects with fatty liver, but matched for BMI, had

increased levels of ceramides (Kolak et al. 2007). These lipids were also increased in visceral AT (VAT) of obese women with IR as compared to healthy lean controls (Choromanska et al. 2019). Similarly, increased ceramides were found in SAT of T2D compared with BMI matched healthy controls (Chaurasia et al. 2016). Moreover, in a cohort of 439 participants, Turpin et al. also reported increased levels of ceramides in WAT alongside the gene expression of the ceramide synthase 6 (CER6), a ceramide synthase isoform responsible for the generation of ceramides with 16 carbons (Turpin et al. 2014). The authors also reported that CerS6-deficient mice when placed on a high-fat diet were protected against the onset of obesity-related IR (Turpin et al. 2014). These studies strongly support the crucial role for ceramides in WAT IR.

Another class of lipids that have been found deregulated in obesity and IR are glycerophospholipids (GPL), a crucial component of the cell membranes and reservoir for the generation of second messengers. Specifically, Pietilainen et al. studied a cohort of monozygotic twin pairs, discordant for body weight (both metabolically healthy) to dissect the WAT lipidomic signature of obesity while ruling out the genetic background; they showed that SAT lipidome of the obese individuals was characterized by an enrichment of PUFA in GPL [palmitoleic and arachidonic acid (AA)] and a reduction of GPL containing shorter and more saturated fatty acids (Pietilainen et al. 2011). By performing both cellular and computational analyses, the authors demonstrated that the increased levels of AA within the GPL fraction was a "healthy" response to the AT enlargement as it preserved the plasma membrane fluidity. On the other hand, when the authors profiled the lipidomic SAT of metabolically impaired obese patients, they observed a generalized reduction of PUFA in the GPL fraction (Pietilainen et al. 2011), which failed to preserve the AT membrane fluidity – a coping mechanism to deal with the AT expansion.

More recently, the importance of the quality of GPL, specifically phosphatidylcholines (PC), was also reported in the AT macrophages (AT-M) (Petkevicius et al. 2019). Specifically, AT-M of obese mice and humans promoted AT inflammation via an enhanced de novo PC biosynthesis, which leads to greater incorporation of SFA and MUFA within the PC fraction (Petkevicius et al. 2019). In an in vitro study, the authors also showed how the inhibition of this pathway in AT-M alleviated obesity-induced WAT inflammation and IR (Petkevicius et al. 2019). Taken together, these data highlight how AT lipidome, alongside other insulin-sensitive tissues, is also greatly remodeled in obesity and IR.

3 Impact of Adipose Tissue Distribution and Ectopic Fat Accumulation on IR

AT distribution (visceral vs subcutaneous) and its ectopic accumulation in organs like liver or muscle are associated with reduced insulin sensitivity. The fatty acid overflow from the AT, due to IR, is the main cause of ectopic fat accumulation and lipotoxicity, i.e., the synthesis/accumulation of toxic lipids that impair metabolic signaling, leading to alteration in glucose and lipid metabolism, IR and impaired

insulin secretion (Gastaldelli and Ferrannini 2014). In fact, the FFA released by the AT in the circulation are also used by other organs including liver, muscle, pancreas, and heart, to accumulate TG in lipid droplets as an energy storage but when in excess this ectopic fat may also promote the synthesis of lipotoxic species like ceramides (see below) (Gaggini et al. 2017; Gastaldelli 2011). Accumulation of intramyocellular TGs (IMTG) has been associated with IR and reduced activation of muscle glycogen synthase (Phillips et al. 1996). However, sarcolemmal, not cytosolic, IMTGs were increased in obese and T2D subjects compared to lean or athletes and associated with IR (Kahn et al. 2021). Interestingly, the improvement in peripheral insulin sensitivity observed after bariatric surgery (biliopancreatic diversion) was associated with the reduction in IMTG (Greco et al. 2002).

Both VAT and intrahepatic triglycerides (IH-TG) are inversely related to insulin-stimulated glucose disposal and this is mainly due to a significant reduction in non-oxidative glucose disposal that in NAFLD is comparable to T2D (Bril et al. 2017; Brouwers et al. 2017; Gastaldelli et al. 2007; Thamer et al. 2007). Similarly, the reduction in glucose clearance during OGTT measured as OGIS or Matsuda index is proportional to IH-TG (Rosso et al. 2016) and VAT (Gastaldelli et al. 2002, 2005) and this is already evident in obese children with NAFLD (Bedogni et al. 2012).

Not only hepatic but also VAT is increased in subjects with NAFLD and is related to higher peripheral IR (Gaggini et al. 2013; Fabbrini et al. 2010a; Kissebah et al. 1982) and glucose intolerance (Gastaldelli et al. 2005, 2007).

However, IH-TG and VAT appear to contribute differently to metabolic function (Gaggini et al. 2013; Fabbrini et al. 2009). Subjects with high IH-TG and high VAT have the highest hepatic, AT, and muscle insulin resistance (Gaggini et al. 2013). These results might indicate that a reduction in VAT should improve peripheral and hepatic insulin sensitivity. Although the decrease in VAT and IH-TG after lifestyle intervention was associated with an improvement in IR in several studies (Thamer et al. 2007; Magkos et al. 2016; Petersen et al. 2005), this improvement was lower in subjects that had higher VAT and IH-TG at baseline (Thamer et al. 2007). However, the surgical removal of omental fat did not improve insulin resistance (Fabbrini et al. 2010b). On the other hand, after pioglitazone treatment adipose tissue distribution showed an increase in SAT and a decrease in VAT, the latter correlated to the decrease in IHTG (Gastaldelli et al. 2021); a similar correlation was observed also in the placebo group where (positive/negative) changes in VAT were equally correlated with (positive/negative) changes in IHTG (Gastaldelli et al. 2021).

4 Obesity and Hepatic Insulin Resistance

Insulin suppresses endogenous glucose production (EGP) as shown during insulin infusion (euglycemic-hyperinsulinemic clamp, EHC). Thus, hepatic insulin resistance is defined as high EGP in presence of high insulin concentrations (at fasting or during insulin stimulation).

After an overnight fasting, the great majority of endogenous glucose is synthesized from gluconeogenic precursors such as lactate/pyruvate, glucogenic amino acids, and glycerol and the rest from glycogenolysis (Landau et al. 1996; Gastaldelli et al. 2000; Roden et al. 2000). In NAFLD, gluconeogenesis (GNG) fluxes tend to be increased as a consequence of increased peripheral lipolysis and protein catabolism that result in increased glycerol and amino acid concentrations (Hyotylainen et al. 2016). Perry et al. showed that insulin suppression of lipolysis also mediates the suppression of hepatic glucose production since this reduces hepatic acetyl-CoA content [an allosteric activator of pyruvate carboxylase (PC)], pyruvate carboxylase (PC) activity and PC flux (Perry et al. 2015). This mechanism also indicates a link between AT and hepatic IR.

Most of the subjects with NAFLD are IR, independently of obesity or diabetes (Bril et al. 2017; Gastaldelli et al. 2007; Rosso et al. 2016; Bugianesi et al. 2005; Yki-Jarvinen 2014; Fabbrini et al. 2009). The result is not only an excessive hepatic glucose production but also a reduced peripheral glucose disposal (Bril et al. 2017; Gastaldelli et al. 2007; Rosso et al. 2016; Bugianesi et al. 2005; Yki-Jarvinen 2014; Fabbrini et al. 2009). Not only obese subjects but also nonobese with hepatic fat accumulation have increased hepatic IR (Bril et al. 2017; Gastaldelli et al. 2007). Hepatic IR increases with the degree of intrahepatic TG accumulation and is already evident even when intrahepatic TGs are less than 5% (Bril et al. 2017; Gastaldelli et al. 2007).

Both total hepatic fat and its composition are associated with the development of IR. It is well established that saturated fatty acids that either comes from the diet or are synthesized from de novo lipogenesis (DNL) are the precursors for the synthesis of lipotoxic lipids like ceramides or diacylglycerols (DAG). De novo synthesized FA (mainly palmitate) might contribute up to 20% of VLDL-TG in insulin resistant subjects (Donnelly et al. 2005). TGs are generally regarded as a safer form of fat storage as compared to more toxic lipids, referred to as lipotoxic species (Listenberger et al. 2003). However, in the long term even simple steatosis is associated with higher risk of mortality (Simon et al. 2020).

Different lipids have been implicated in the onset of hepatic IR (Samuel and Shulman 2018), but the exact mechanism is still debated. Hepatic ceramides and DAG were associated with severe forms of NAFLD/NASH and hepatic IR (Samuel and Shulman 2012; Luukkonen et al. 2016; Puri et al. 2007; Mota et al. 2016). It has been hypothesized that excess DAG might be implicated in the development of IR in liver and muscle through the activating of PKCε that binds to insulin receptor and inhibits its tyrosine kinase activity interfering with the ability of insulin to phosphorylate IRS-1 (Mack et al. 2008) and IRS-2 (Postic and Girard 2008; Szendroedi et al. 2014; Samuel et al. 2007) (Fig. 1). Both muscle (Szendroedi et al. 2014) and liver (Ter Horst et al. 2017) cytosolic DAG were increased in IR and correlated with activation of PKCε. However, the relationship between PKCε and IR has been questioned by Brandon et al. (2019) that showed that global and adipose tissue deletion of PKCε protects against diet-induced glucose intolerance and while mice with liver-specific deletion of PKCε (LEpsKO) had peripheripheral and hepatic insulin sensitivity in LEpsKO similar to contols.

5 Obesity and Muscle Insulin Resistance

Muscle IR is a condition very common in subjects with obesity and has a large effect on whole-body glucose turnover. During fasting muscle oxidizes mainly fatty acids. In condition of euglycemia-hyperinsulinemia muscle is the major site for glucose disposal (70–80% of glucose infused) while in postprandial state (with hyperglycemia and hyperinsulinemia) only 25–30% of ingested glucose is taken up by skeletal muscle. About half of the glucose taken up by the muscle after a meal is oxidized, 35% is stored as glycogen and 15% utilized for glycolysis that results in the production and release of lactate, alanine, or pyruvate (Kelley et al. 1988; Mitrakou et al. 1990).

Muscle IR is due to defects in insulin signaling with reduced phosphorylation and glucose transport (Petersen and Shulman 2018). Insulin binds to insulin receptors and initiates the intracellular signaling events that promote the translocation of glucose transporter 4 (GLUT4) to the plasma membrane, which is necessary for glucose transport into the cell. Subjects with severe IR, like those with family history of T2D or already diabetics, have impaired postprandial muscle insulin signaling (Cusi et al. 2000; Krook et al. 2000) with decreased glucose oxidation and storage, and a greater release of glycolytic metabolites (Mitrakou et al. 1990) that in turn are used by the liver for gluconeogenesis. Indeed, subjects with peripheral IR often have also hepatic IR.

Also, AT IR and increased lipolysis are common in subjects with muscle IR. Excess circulating fatty acids also impair muscle insulin signaling and glucose metabolism (Boden and Shulman 2002). Thus, obesity and AT IR are strongly related to peripheral IR. Moreover, the observation that offspring of type 2 diabetic patients are highly insulin resistant in the muscle and at higher risk of developing diabetes, especially if obese, indicates that muscle IR is probably genetically driven.

As with the liver, ectopic fat accumulation promotes the development of skeletal muscle IR, but the contribution of the different lipid species in human obesity is still debated. Different studies have coherently reported the causative role of ceramides in skeletal muscle IR (Adams et al. 2004; Bourbon et al. 2002; Coen et al. 2010; Holland et al. 2007). Indeed, muscle biopsies of mice and subjects with IR display increased ceramide levels (Turpin et al. 2014; Adams et al. 2004; Coen et al. 2010), and in mice fed a high-fat diet the administration of inhibitors of ceramide biosynthesis, such as myriocin, improved their insulin sensitivity (Kurek et al. 2015) . Moreover, in a study where participants were overfed with a diet enriched in SFA, Lukkonen et al. observed an increased IR, intrahepatic fat accumulation paralleled by increased plasma ceramides (Luukkonen et al. 2018). The mechanism by which ceramides promote IR is based upon their capability to inhibit the protein kinase B (Akt) via two distinct mechanisms as summarized in Fig. 1. In the first model, ceramides activate the PKA isoform zeta (ζ) which phosphorylates Akt thus impairing its translocation to the plasma and subsequent insulin signaling actions (Powell et al. 2003; Stratford et al. 2001). In the second model, ceramides activate protein phosphatase 2A (PP2A), which leads to dephosphorylation and subsequent inactivation of Akt (Chavez et al. 2003). Some evidence has also indicated that DG

can promote skeletal muscle IR (Szendroedi et al. 2014; Itani et al. 2002) but this has been questioned since muscle DG was not correlated with IR in different studies (Anastasiou et al. 2009; Coen et al. 2013; Amati et al. 2011).

6 Conclusions

In this chapter, we reviewed the metabolic basis of obesity-related IR and discussed the importance of AT composition and distribution as a driver of whole-body IR, alongside a description of the lipid remodeling occurring in insulin-sensitive tissues during these changes. The wealth of knowledge generated over the last decades of research has increased our understanding of the mechanism behind the obesity-driven IR. However, several mechanistic aspects require further investigation. A deeper understanding of these mechanisms would aid in finding targeted strategies to improve patient's health.

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Obesity, Senescence, and Senolytics

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Contents

Abstract

Obesity is a major risk factor for the development of comorbidities such as type 2 diabetes, neurodegenerative disorders, osteoarthritis, cancer, cardiovascular and renal diseases. The onset of obesity is linked to an increase of senescent cells within adipose tissue and other organs. Cellular senescence is a stress response that has been shown to be causally linked to aging and development of various age-related diseases such as obesity. The senescence-associated-secretory phenotype of senescent cells creates a chronic inflammatory *milieu* that leads to local and systemic dysfunction. The elimination of senescent cells using pharmacological approaches (i.e., senolytics) has been shown to delay, prevent, or alleviate obesity-related organ dysfunction.

Keywords

Adipose tissue · Cellular senescence · Obesity · SASP · Senolytics

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1 Introduction

The worldwide prevalence of obesity is steadily increasing and has nearly tripled since 1975. The WHO estimated that in 2016, 39% and 13% of the world's adult population was overweight (BMI $\geq$25) or obese (BMI $\geq$30), respectively. Obesity is a major risk factor for developing multiple chronic diseases, such as type 2 diabetes, osteoarthritis, cancer, neurodegenerative disorders, osteoarthritis, cardiovascular and renal dysfunction, among others (Kahn and Flier 2000). Obesity results in the induction and accumulation of senescent cells in various organs such as adipose tissue, liver, brain, and pancreas. Obesity is associated with a reduction in health/lifespan and can be considered to be an accelerated aging-like state in some respects (Ahima 2009). Interestingly, transplanting senescent preadipocytes into young mice recapitulates this accelerated aging phenotype (Xu et al. 2018). Interventions that extend health and lifespan are linked to decreased adiposity, including calorie restriction and exercise (Fontana and Klein 2007; Schafer et al. 2019). Cellular senescence is a physiological stress response that has been found to be induced by obesity-mediated pathological alterations. Accumulation of senescent cells has been shown to affect the function of neighboring cells, is linked to low-grade chronic inflammation, and promotes carcinogenesis through the secretion of cytokines and chemokines, commonly referred to as the senescence-associated secretory phenotype (SASP) (Acosta et al. 2013; Alimirah et al. 2020; Gonzalez-Meljem et al. 2018; Iske et al. 2020; Palmer et al. 2019b; Tchkonia et al. 2010; Xu et al. 2018). Interestingly, the elimination of senescent cells genetically or pharmacologically in animal models using senolytics leads to alleviation of obesity-related tissue dysfunction and comorbidities associated with obesity, illustrating that cellular senescence may be causally linked to the obesity phenotype and progression (Kirkland and Tchkonia 2020; Palmer et al. 2015; Palmer et al. 2019b; Tchkonia et al. 2021; Xu et al. 2015).

2 Obesity

Obesity has been shown to promote an accelerated aging-like phenotype and reduce overall lifespan and health span. Obesity increases risk for onset of age-related diseases and comorbidities including diabetes, hypertension, cancer, cognitive dysfunction, atherosclerosis, and vascular dysfunction (Ahima 2009; Björntorp 1990; Colditz et al. 1995; Lean 2000; Minamino et al. 2009; Tchkonia et al. 2010). Interventions that result in a decrease in adipose tissue mass have been shown to increase health and life span. These include calorie restriction or surgical removal of visceral fat (Barzilai and Gupta 1999; Masoro 2006; Muzumdar et al. 2008). Conversely, transplantation of senescent preadipocytes into younger mice leads to physical dysfunction and a reduction of health and lifespan (Wang et al. 2020; Xu et al. 2018).

3 Adipose Tissue

Adipose tissue size and distribution changes throughout life (Bazzocchi et al. 2013). In obese subjects, adipose tissue can be the largest organ in the body. Over half of the body mass in women with body mass index (BMI) >40 is adipose tissue. Adipose tissue stores energy and is a major endocrine organ. Adipose tissue is proximal to almost all other organs and acts a protective buffer against mechanical and molecular insults, such as trauma or lipotoxicity. Obesity results in extensive alterations in adipose tissue, with an increase in proinflammatory cytokines (Mancuso and Bouchard 2019). The increase in adipose tissue in obese subjects occurs through two cellular processes: expansion of adipocyte size and/or number. On the one hand, hyperplastic expansion results from an increase in adipocyte number though enhanced differentiation of preadipocytes (also called adipose-derived stem cells or mesenchymal stem cells) into adipocytes. On the other, hypertrophic expansion results from enlargement of existing adipocytes (Cartwright et al. 2007; Fried and Kral 1987). In mild obesity, fat cell size is predominantly increased, whereas in cases of severe obesity, adipocyte numbers are also increased (Cinti et al. 2005; Lacasa et al. 2007; Shillabeer et al. 1990; Strissel et al. 2007). Adipose tissue can be divided into subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT), which differ in gene expression profiles (Lakowa et al. 2015; Lefebvre et al. 1998; Villaret et al. 2010). During aging and the early stages of obesity, the proportion of VAT increases relative to SAT, and this is associated with insulin resistance (Matsuzawa et al. 1995). Different fat depots are distinct in terms of their relative contributions to proinflammatory cytokines and disease severity in obesity and aging. In particular, increased VAT has been shown to be an important mediator of metabolic disease progression and lipotoxicity (Carr et al. 2004; Tchkonia et al. 2013a; Thomou et al. 2010; Wannamethee et al. 2007). Increased VAT mass results in higher risk for diabetes and increased mortality (Pischon et al. 2008). Surgical removal of VAT leads to an increase in insulin sensitivity (Barzilai and Gupta 1999; Gabriely et al. 2002; Huffman and Barzilai 2009). Another important difference between fat depots is that proinflammatory cytokine and chemokine production is higher in VAT than SAT. Moreover, high fat diets increase the already high expression in VAT of proinflammatory cytokines, such as TNFα and PAI1, even further (Einstein et al. 2005; Huffman and Barzilai 2009; Starr et al. 2009; Thomou et al. 2010).

4 Cellular Senescence

Cellular senescence is a stress response that leads to a terminal proliferative arrest. Cellular senescence has been shown to be an important tumor suppressive mechanism. In addition, cellular senescence has important functions during embryonic development and tissue repair (Demaria et al. 2014; Hayflick and Moorhead 1961; Jeyapalan and Sedivy 2008; Narita and Lowe 2005). Besides these beneficial functions of cellular senescence, accumulation of senescent cells is associated with various conditions such as obesity, diabetes, cardiovascular dysfunction,

osteoporosis, neurodegeneration, and fibrosis (Muñoz-Espín and Serrano 2014). Recent findings indicate that cellular senescence is causally linked to progression of these diseases, making it a promising therapeutic target (Farr et al. 2017; Musi et al. 2018; Roos et al. 2016; Schafer et al. 2017). Various stressors have been shown to induce senescence, including oncogenic signaling, DNA-damage, and metabolic-insults (Muñoz-Espín and Serrano 2014). Induction of senescence can involve upregulation of cell cycle inhibitors through the p53/p21 pathway and/or the p16/pRB pathway (Hernandez-Segura et al. 2018). Moreover, senescent cells can display several features, such as an increase in senescence-associated β-galactosidase (SA-β-gal) in some, but not all senescent cells (Dimri et al. 1995). The phenotypes that senescent cells exhibit have been shown to be dependent on the type of stressor causing senescence, originating cell-type, and time since induction of senescence, which may take days to weeks to establish fully (at least in vitro) (Kuilman et al. 2008; Kuilman and Peeper 2009; Passos et al. 2010). The SASP of senescent cells can include a plethora of proinflammatory cytokines, chemokines, extracellular matrix remodelers, bioactive lipids (bradykines, ceramides, prostanoids), noncoding nucleotides (microRNAs, mitochondrial DNA), and metabolites, including reactive oxygen species (ROS) (Coppé et al. 2010; Krtolica and Campisi 2002; Parrinello et al. 2005; Wiley et al. 2016; Xue et al. 2007). Mediated by the SASP, senescent cells can remodel their local microenvironment and interact with neighboring cells in a paracrine manner. This leads to a proinflammatory *milieu*, spread of the senescent phenotype to neighboring cells, recruitment of immune cells, and paracrine alteration of cellular functions (Sedelnikova et al. 2007; Suganami et al. 2005). Senescent cells are resistant to apoptosis and are usually cleared by the immune system (Prata et al. 2018; Wang 1995). The accumulation of senescent cells during aging and various diseases has been shown to be an important factor contributing to disease severity and progression. Senescent cell accumulation and therefore high levels of SASP factor secretion appear to contribute to the chronic low-grade inflammation in obesity (De Pergola and Pannacciulli 2002; Fried et al. 1998; Hotamisligil and Spiegelman 1994; Loffreda et al. 1998; Perreault and Marette 2001; Samad and Loskutoff 1996; Samad et al. 1999; Sartipy and Loskutoff 2003; Stefan et al. 2002; Tchkonia et al. 2013b; Vgontzas et al. 1997; Visser et al. 1999; Xu et al. 2003). Through their SASP, senescent cells are not only able to alter the microenvironment but also to have a systemic impact on physiological function.

5 Cellular Senescence in Obesity

Adipose tissue senescent cell number can increase with aging and in obesity (Conley et al. 2020; Espinosa De Ycaza et al. 2021; Justice et al. 2018; Ogrodnik et al. 2019; Palmer et al. 2019b; Schafer et al. 2016; Tchkonia et al. 2013b; Xu et al. 2015). These senescent cells are primarily located in visceral adipose tissue, but also subcutaneous depots (Escande et al. 2014; Espinosa De Ycaza et al. 2021; Palmer et al. 2019b; Rouault et al. 2021). The main cell types undergoing senescence in

obesity are adipocyte progenitor cells (preadipocytes) and endothelial cells. Preadipocytes make up 15–50% of cells within adipose tissue and have been shown to undergo cellular senescence during chronological aging and in various pathologies such as obesity and diabetes (Minamino et al. 2009). Cells isolated from obese human and rat adipose tissue exhibit more SA-β-gal positivity than their lean counterparts (Tchkonia et al. 2010). In addition, BMI correlates positively with adipose tissue SA-β-gal activity and p53 activation (Conley et al. 2020). Moreover, in obesity, endothelial cells have upregulated cellular senescence markers (Villaret et al. 2010). There is also accumulation of senescent $CD4^+$ T-cells and macrophages in VAT in a diet-induced obese mouse model (Shirakawa et al. 2016). Interestingly, senescent cells not only accumulate within adipose tissue in obesity, but also distant organs such as the brain. In HFD mice, senescent glial cells accumulate around the third ventricle (Ogrodnik et al. 2019). This is consistent with the observation that senescent cells can spread their phenotype in an endocrine manner to distant organs, resulting in systemic effects (Keyes et al. 2005; Xu et al. 2018). Senescent preadipocytes are unable to differentiate into fully functional adipocytes when stimulated with adipogenic stimuli (Mitterberger et al. 2014; Palmer et al. 2019a; Palmer et al. 2019b; Tchkonia et al. 2010; Xu et al. 2015). The SASP of preadipocytes has been shown to block adipogenesis and to attract immune cells (especially macrophages) that can worsen insulin resistance (Xu et al. 2015). In very obese subjects, senescent cell burden can be as much as 30-fold higher than in non-obese counterparts. More studies are needed to fully characterize cellular senescence within adipose tissue. The lack of a universal marker of cellular senescence and the general heterogeneity of senescent cell populations make it difficult to determine precisely the extent of senescence (Hernandez-Segura et al. 2018). For example, macrophages have been shown to have upregulated classical markers of senescence, including p16 and SA-β-gal, even though it is not clear if they are truly senescent (Liu et al. 2019).

6 Inducers of Cellular Senescence in Obesity

Obesity is associated with chronic stress that can induce senescence as well as an increase in circulating SASP factors, bioactive metabolites (ROS, lipids), growth factors, and signaling molecules. The high glucose levels that are common in obese subjects can induce cellular senescence in a variety of different cell types, such as preadipocytes, renal mesangial, endothelial, and epithelial cells, and fibroblasts (Blazer et al. 2002; Chen et al. 2019; Cramer et al. 2010; Kuki et al. 2006; Mortuza et al. 2013; Palmer et al. 2019a, 2019b, 2021; Yokoi et al. 2006). Several mechanisms have been proposed to explain how high glucose induces cellular senescence, such as increased ROS production or activating PI3K/AKT signaling, which can induce senescence through mTOR activation and FOXO inhibition (Huy et al. 2018; Mortuza et al. 2013; Nogueira et al. 2008; Sheu et al. 2005). Obese patients have increased levels of fatty acids; chronic exposure of cells to these fatty acids can contribute to lipotoxicity (Björntorp et al. 1969). Furthermore, ceramide

synthesis is increased in obesity and high levels ceramide levels can induce cellular senescence (Jadhav et al. 2013; Venable et al. 1995; Venable and Yin 2009). ROS are a potent inducer of cellular senescence and can lead to extensive DNA damage. Furthermore, ROS can damage proteins, lipids, and other macromolecules. Oxidative stress is more pronounced in obese subjects who have increased levels of oxidized low-density lipoproteins (ox-LDL). In addition, ox-LDL can induce endothelial progenitor cell senescence (Hurtado-Roca et al. 2017; Imanishi et al. 2004). Mitochondria are important for general homeostasis and bioenergetic metabolism. Obesity induces mitochondrial dysfunction, while caloric restriction can alleviate mitochondrial dysfunction (de Mello et al. 2018; Lanza et al. 2012). Obesity-related mitochondrial dysfunction can entail a decrease in adipocyte mitochondrial oxidative capacity (de Mello et al. 2018). Interestingly, mitochondrial dysfunction can induce senescence with a distinct SASP that inhibits adipogenesis (Wiley et al. 2016). Hyperplasia in obesity goes hand-in-hand with increased preadipocyte replicative history. Consistent with this, short telomeres correlate with adiposity and telomeric attrition, in turn, can induce senescence (Lee et al. 2011).

7 Implications of Cellular Senescence in Obesity

Senescent cells are heterogenous and differ in their proinflammatory phenotypes. Thirty to seventy percent of senescent cells can acquire a profound proinflammatory phenotype and secrete large amounts of TNFα, ROS, interleukin (IL)-1α, IL-6, IL-8, interferon-γ, and metalloproteinases, as well as exosomes, microRNAs, and other noncoding RNAs (Coppé et al. 2010; Lei et al. 2017; Nelson et al. 2018; Tchkonia et al. 2021; Terlecki-Zaniewicz et al. 2018) (unpublished observations). Through their SASP, senescent cells can alter their local microenvironment, change the phenotype of surrounding cells, and amplify proinflammatory signaling cascades. In co-culture conditions without direct cell–cell contact between preadipocytes and macrophages, a proinflammatory phenotype is induced in the preadipocytes with production of TNFα (Suganami et al. 2005). TNFα can, by itself, induce cellular senescence in preadipocytes, endothelial cells, and fibroblasts (Tchkonia et al. 2010). Conditioned media from senescent preadipocytes can induce senescence in a paracrine manner in vascular smooth muscle cells and endothelial cells, leading to increased expression of proinflammatory SASP factors (Parvizi et al. 2021). Another mechanism that amplifies the senescent phenotype is paracrine induction of senescence through the generation of ROS and SASP factors (Acosta et al. 2013; Nelson et al. 2018). Senescent cells also attract and activate immune cells such as macrophages and neutrophils (Hickson et al. 2019; Lagnado et al. 2021; Palmer et al. 2019b; Prata et al. 2018; Yamada et al. 2018). This chronic proinflammatory *milieu* impairs the function of neighboring cells. For example, adipose progenitor cells from obese patients have reduced adipogenic capacity (Conley et al. 2020; de Girolamo et al. 2013; Gustafson et al. 2019). The exposure of preadipocytes to the proinflammatory microenvironment created by senescent cells impairs their adipogenesis in a paracrine manner through secretion of activin A, TNFα, and

IL-6 by the senescent cells (Mitterberger et al. 2014; Xu et al. 2015; Zaragosi et al. 2010). This impairment of adipogenesis limits lipid storage and induces ectopic lipid accumulation, with deposition in the liver and skeletal muscle, contributing to insulin resistance (Hammarstedt et al. 2018; Listenberger et al. 2003; Tchkonia et al. 2010; Wang et al. 2008). Obesity also affects the gut microbiome, inducing the production of DNA-damaging and senescence-inducing metabolites, potentially contributing to the development of hepatocellular carcinoma (Yoshimoto et al. 2013). Conversely, clearing senescent cells can alleviate intestinal inflammation and modulate the microbiome, at least in aged mice (Saccon et al. 2021). Cellular senescence in obesity not only has local consequences, but also can be associated with widespread, detrimental, long-lasting effects.

8 Senolytic Therapies in Obesity

Eliminating senescent cells appears to be a promising approach for extending health span and mitigating disease progression in various animal models. The first reports of clinical trials seem to recapitulate these findings. The first pharmacological approaches for eliminating senescent cells are based on blocking the networks and pathways that senescent cells use to protect themself from their own pro-apoptotic SASP (Chang et al. 2016; Fuhrmann-Stroissnigg et al. 2017; Yousefzadeh et al. 2018; Zhu et al. 2015, 2016, 2017). These senescent cell anti-apoptotic pathways (SCAPs) allow senescent cells to evade apoptosis. Senolytic therapies transiently inhibit this protective mechanism, making senescent cells susceptible undergoing apoptosis in response to their own pro-apoptotic SASP (Kirkland and Tchkonia 2020; Zhu et al. 2015). Using this mechanism-based approach, a number of senolytics have been identified, including Dasatinib plus Quercetin (D/Q) and Fisetin, which are currently in clinical trials for various age-related pathologies (Kirkland and Tchkonia 2020). Beneficial effects of senescent cell elimination in obesity have been illustrated using genetic approaches in mice and are recapitulated by senolytics (Palmer et al. 2019b). The *INK-ATTAC* transgenic mouse model allows selective depletion of $p16^{Ink4a}$ highly expressing cells (Baker et al. 2011; Xu et al. 2015). In obesity, elimination of senescent cells by genetic or pharmacological means (i.e., senolytics) led to an increase in metabolic health (Palmer et al. 2019b; Xu et al. 2015). In vitro treatment of human adipose tissue explants with D/Q caused elimination of senescent cells and a decrease in proinflammatory SASP markers, including IL-6, IL-8, GM-CSF, and MCP-1. Furthermore, PPARγ and CEBPα levels were increased, suggesting an increase in adipogenic potential (Xu et al. 2018). Remarkably, preliminary data in patients with diabetic kidney disease receiving D/Q indicated a significant reduction of senescent cell burden in adipose tissue (Hickson et al. 2019). The pathological changes mediated by obesity may accelerate continuous and systemic senescent cell generation. The effectiveness of the brief administration schedule of senolytics and resulting beneficial effects suggest “hit and run” senolytic regimens may be feasible. Brief exposure to senolytics appears to cause senescent cell apoptosis and return of senescent cells

takes time, reducing the risk of side effects, meaning that weekly or monthly administration of senolytics with a short elimination half-life could be effective. Targeting cellular senescence, a root cause contributor to disease progression, in obesity could be a promising strategy for mitigating the health burden of obesity. More bench research and clinical studies are necessary to confirm these promising findings about senolytic therapy in obesity, diabetes, and associated comorbidities.

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Obesity and Obstructive Sleep Apnea

Maria R. Bonsignore

Contents

Abstract

Obstructive sleep apnea (OSA) is characterized by upper airway collapse during sleep. Chronic intermittent hypoxia, sleep fragmentation, and inflammatory activation are the main pathophysiological mechanisms of OSA. OSA is highly prevalent in obese patients and may contribute to cardiometabolic risk by exerting detrimental effects on adipose tissue metabolism and potentiating the adipose tissue dysfunction typically found in obesity. This chapter will provide an update on: (a) the epidemiological studies linking obesity and OSA; (b) the studies exploring the effects of intermittent hypoxia and sleep fragmentation on the adipose tissue; (c) the effects of OSA treatment with continuous positive airway pressure (CPAP) on metabolic derangements; and (d) current research on new anti-diabetic drugs that could be useful in the treatment of obese OSA patients.

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Cardiovascular outcomes · Dyslipidemia · Insulin resistance · Microbiome · NAFLD · Sympathetic activation · Type 2 diabetes

1 Introduction

Obstructive sleep apnea (OSA) is a complex disorder characterized by partial or complete collapse of upper airway during sleep (Lévy et al. 2015). During obstructive respiratory events, there are progressive respiratory efforts, associated with development of hypoxia and hypercapnia and sympathetic activation. An arousal from sleep usually re-establishes airway patency, but the respiratory events recur on return to sleep. OSA severity is commonly assessed based on the frequency of respiratory events during sleep (Apnea Hypopnea Index, AHI); OSA is defined as mild, moderate, or severe for AHI between 5 and <15/h, 15 and <30/h, and ≥30/h, respectively. The traditional OSA metrics are currently debated, since the frequency of respiratory events poorly reflects the clinical aspects and the prognosis of OSA (Pevernagie et al. 2020; Malhotra et al. 2021). Other severity markers are the frequency of oxygen desaturation (Oxygen Desaturation Index, ODI), mean and lowest oxygen saturation (SpO_2) during sleep, and time spent at $SpO_2 < 90\%$ during sleep. The nocturnal hypoxic burden has been recently proposed as a prognostic marker of OSA severity (Azarbarzin et al. 2019, 2020).

The main symptoms of OSA are intermittent snoring, daytime sleepiness, fatigue, and nonrestorative sleep. OSA is associated with major comorbidities such as diabetes, hypertension, dyslipidemia or chronic kidney disease (Bonsignore et al. 2019), and with a high cardiovascular morbidity and mortality (Xie et al. 2017). A strong relationship has been repeatedly shown between OSA and hypertension, at least partly mediated by hyperactivity of the carotid body, which in turn increases sympathetic nerve activity (Badoer 2020).

The main treatment of OSA is continuous positive airway pressure (CPAP) applied through a nasal or facial mask during sleep to maintain upper airway patency (Lévy et al. 2015). CPAP treatment is indicated in patients with excessive daytime sleepiness irrespective of OSA severity, and in moderate-severe OSA. Randomized controlled studies in non-sleepy OSA patients with coronary artery disease have not shown consistent and/or significant effects of CPAP treatment on cardiovascular morbidity or mortality, suggesting the opportunity to modify the epidemiologic approach and better identify subgroups at high risk (Pack et al. 2021). Different clinical and physiological phenotypes exist (Zinchuk and Yaggi 2020; Bailly 2021), confirming that OSA pathophysiology is heterogeneous. Precision medicine aims at selectively targeting the major aspects of OSA (Randerath et al. 2018), and new treatment approaches are being developed according to OSA phenotypes (Eckert 2018).

The relationship between OSA and obesity has been explored both clinically and in cell and animal models, since a dangerous synergistic effect has been

hypothesized with special regard to the effects of intermittent hypoxia on adipose tissue. Hypoxia exerts differential effects in adipose tissue according to the sustained or intermitted modality of exposure (Gozal et al. 2017), and intermittent hypoxia is associated with adipose tissue dysfunction (Ryan et al. 2019). Moreover, both OSA and obesity are associated with systemic pro-inflammatory changes and increased cardiovascular risk. Hypoxia, however, is not the only factor involved in the OSA-obesity relationship. A link exists between obesity and insufficient sleep in both adults (Zhou et al. 2019) and children (Sun et al. 2020), and preliminary data indicate that sleep extension may counteract dysmetabolic changes associated with short sleep (Henst et al. 2019). Since OSA causes sleep fragmentation (Lévy et al. 2015), it might also interact with obesity through sleep disruption (Poroyko et al. 2016). Finally, the effects of CPAP treatment have been studied to assess whether resolution of OSA might positively modify cardiometabolic variables.

Extensive reviews on the metabolic aspects of OSA have been published (Bonsignore et al. 2012; Trzepizur et al. 2018; Ryan et al. 2019, 2020). This chapter aims to provide an update on: (a) the epidemiological and clinical studies on obesity and OSA; (b) the studies exploring the effects of intermittent hypoxia and sleep fragmentation on the adipose tissue; c) the effects of CPAP treatment; and (d) the possible use of new anti-diabetic drugs in obese OSA patients.

2 Epidemiology

The association of OSA and visceral obesity has been recognized over 20 years ago (Vgontzas et al. 2000). Visceral obesity is associated with increased collapsibility of upper airways, at least partly explained by the deposition of fat in the neck and reduced traction on the airways in the supine position (Drager et al. 2013). The worsening of the obesity epidemic worldwide is a major factor in the progressive increase in OSA prevalence (Young et al. 2002; Peppard et al. 2000, 2013). According to recent estimates, OSA affects about a billion people worldwide when an AHI >5/h is considered as cut-off for OSA diagnosis, or about half a billion people if only moderate-severe OSA, i.e. AHI > 15, is considered (Benjafield et al. 2019). OSA prevalence is higher in men than in women and increases with age (Young et al. 2002; Peppard et al. 2013). The lower prevalence in the female sex might partly be explained by the peripheral distribution of fat in females, especially in the premenopausal period (Mazzuca et al. 2014).

A close relationship has been shown between AHI and weight changes. A longitudinal study in the Wisconsin Sleep Cohort found that a 10% weight gain predicted a 32% increase in AHI, and a 10% weight loss predicted a 26% decrease in AHI (Peppard et al. 2000). Nonrestorative sleep was also associated with increased BMI in longitudinal studies (Reither et al. 2021). While weight loss is highly recommended in OSA patients (Hudgel et al. 2018), lifestyle interventions produced variable results and may not completely resolve OSA (Araghi et al. 2013). The positive effects of lifestyle intervention on OSA persisted during long-term follow-up, despite some increase in weight in both diabetic (Kuna et al. 2021) and

nondiabetic patients (Tuomilheto 2014). Persistent weight loss requires a change in lifestyle which is often a problematic target (Xanthopoulos et al. 2018). Pursuing weight loss in OSA patients remains a major goal, since it decreases AHI and possibly cardiometabolic risk (Edwards et al. 2019; Carneiro-Barrera et al. 2019). In addition, high level of physical activity was recently reported to exert an independent protective effect against the development of OSA (Liu et al. 2021).

OSA is often associated with the metabolic syndrome (Xu et al. 2015) which is diagnosed clinically based on three out of five cardiometabolic risk factors, i.e., waist circumference, blood pressure, fasting glucose, serum triglyceride, and HDL-cholesterol. The metabolic syndrome is a clinical marker of insulin resistance (IR), and experimental and clinical data support a causal relationship between OSA and IR (see reviews by Bonsignore et al. 2012; Drager et al. 2013; Ryan et al. 2020). In prospective studies, OSA was a risk factor for incident metabolic syndrome (Hirotsu et al. 2018), and metabolic syndrome carried an increased risk of incident OSA (Kim et al. 2021). Obesity is a major confounder in the OSA-metabolic syndrome interaction (Wakabayashi et al. 2018). Insulin resistance is a pre-diabetic condition; accordingly, OSA severity was associated with increasing levels of glycosylated hemoglobin in nondiabetic patients (Kent 2014a) and increasing prevalence of type 2 diabetes (Kent et al. 2014b) in cross-sectional studies. A high risk for incidence of type 2 diabetes was found in moderate-severe OSA (Wang et al. 2013; Qie et al. 2020). Therefore, obesity and OSA may potentiate each other in causing dysmetabolism (Aurora and Punjabi 2013; Pugliese et al. 2020).

Nocturnal intermittent hypoxia (IH) was shown to be associated with IR in several studies (reviewed in Bonsignore et al. 2012). Recent work examined the impact of sleep quality on IR. A decreasing duration of slow-wave sleep was associated with progressive worsening of IR in OSA patients (Huang et al. 2021). Short sleep duration was also found independently associated with poor glycemic control (Siwasaranond et al. 2016), especially when the amount of slow-wave sleep decreased (Ahn et al. 2021). One study found that REM sleep AHI, but not non-REM AHI, was associated with worsening of HbA1c levels in diabetic patients, perhaps reflecting higher sympathetic activation in REM sleep (Grimaldi et al. 2014). Experimental sleep fragmentation for two nights in healthy subjects decreased insulin sensitivity, and increased morning cortisol level and sympathetic activity (Stamatakis and Punjabi 2010). In Black subjects from the general population, poor nocturnal sleep and markers of OSA severity were positively associated with fasting glucose, HbA1c, and IR (Yano et al. 2020). In OSA patients AHI and sleep fragmentation, expressed as microarousal index, correlated with metabolic variables (Zhao et al. 2019). On the other hand, an improved sleep quality was associated with metabolic improvement in OSA patients starting CPAP treatment (Magnusdottir et al. 2021).

OSA may adversely influence health in patients with type 2 diabetes. In the Nagahama study, moderate/severe sleep disordered breathing (SDB) was associated with increased risk of diabetes only in women, irrespective of the menopausal status, suggesting a sex-related difference. In the same study, SDB mediated about 20% of the association of obesity and diabetes or systemic hypertension

(Matsumoto et al. 2018). In a study in the Korean general population, OSA in non-obese subjects was associated with a high risk of diabetes and impaired glucose tolerance, whereas no association was found in obese subjects (Kim et al. 2013). In patients with poorly controlled diabetes, nocturnal IH was associated with worse glycemic control independent of obesity (Torrella et al. 2015). A longitudinal study found that incidence of cardiovascular and microvascular complications in diabetic patients with OSA was higher than in controls (Adderley et al. 2020). Daytime sleepiness may be a marker of OSA severity as it was associated with worse glycemic control in male OSA patients with type 2 diabetes and BMI < 35 kg/m^2 (Aurora and Punjabi 2019). Moreover, OSA is often associated with diabetic complications. Nocturnal hypoxemia and sleep fragmentation may delay healing of diabetic ulcers (Maltese et al. 2018; Chen et al. 2021) and increase the risk of severe vascular disease (Stadler 2018) or diabetic kidney disease (Leong et al. 2016). Severe retinopathy was associated with occurrence of OSA and its development might be slowed by CPAP treatment (Altaf et al. 2017). Therefore, besides the association of OSA with diabetes and poor glycemic control, some data also suggest a negative impact of OSA on the clinical course of diabetes.

The relationship between OSA and non-alcoholic fatty liver disease (NAFLD) is of high clinical interest. NAFLD is a multifactorial disease often associated with obesity and metabolic syndrome (Kumar et al. 2020). NAFLD is characterized by accumulation of fat in $\geq$5% of hepatocytes in the absence of significant alcohol consumption or secondary causes of hepatic steatosis. Its spectrum includes simple steatosis, non-alcoholic steatohepatitis (NASH), advanced fibrosis, and an increased risk for hepatocellular carcinoma, cirrhosis, and liver failure. A cross-sectional ICD code-based analysis reported a higher prevalence of OSA in subjects with NAFLD compared with controls (15.9 vs 8.9%) (Renno et al. 2021). A recent longitudinal study from Korea found increased incidence of OSA in patients with NAFLD, and a linear positive relationship between OSA incidence and the fatty liver index (Chung et al. 2021). Patients with OSA showed increased alanine transferase (ALT) level and prevalence of NAFLD (Jin et al. 2018), and OSA was associated with an advanced stage of biopsy-proven NAFLD (Musso et al. 2013). The association of NAFLD and OSA was especially frequent in morbidly obese subjects, and OSA severity correlated with NAFLD severity (Aron-Wisnewsky et al. 2012; Benotti et al. 2016; Schwenger et al. 2020); moreover, the histologic evidence of NAFLD and liver fibrosis in morbidly obese patients with OSA correlated with the degree of nocturnal hypoxemia (Polotsky et al. 2009). Increased serum lysyl oxidase (LOX) level, which is involved in the pathogenesis of liver fibrosis, might be used as a biomarker of NAFLD in obese OSA patients (Mesarwi et al. 2015). After metabolic bariatric surgery, severity of both OSA and NAFLD decreased (Zhang et al. 2020).

Dyslipidemia is frequent in OSA patients (Nadeem et al. 2014) and may be worsened by obesity (Karkinski et al. 2017). The pathogenesis of dyslipidemia is highly complex, since IH and oxidative stress may act in both the adipose tissue and the liver and affect multiple steps of lipid metabolism through different mechanisms (Barros and García-Río 2019). A recent study found that acute IH increased post-prandial plasma triglycerides (TG) in both OSA patients and normal subjects, but

differently from control subjects OSA patients showed persistently elevated TG levels (Morin et al. 2021). Fractional clearance rate of TG and cholesteryl ester was slower in OSA patients than age- and BMI-matched controls (Drager et al. 2018). In patients with type 2 diabetes and OSA, increased lipolysis and decreased antilipolytic effects of alpha 2-adrenergic stimulation were found to be associated with OSA severity (Trinh et al. 2021). Metabolism of free fatty acids (FFA) was also shown to be altered in OSA patients, mainly through adipocyte IR and severity of nocturnal hypoxemia (Stefanovski et al. 2020). Clinical observational and randomized controlled studies have not provided a unique picture (see Barros and García-Río 2019 for review). Results of recent epidemiological studies support the association of OSA with dyslipidemia. In middle-aged nondiabetic subjects, OSA was independently associated with higher total cholesterol levels (Silva et al. 2021). In a large study in subjects with suspected OSA, AHI and nocturnal IH were associated with higher TG and lower HDL-cholesterol (Trzepizur et al. 2013). Similar results were reported in subjects from the European Sleep Apnea Database (ESADA) cohort (Gündüz et al. 2018, 2019). Plasma lipids were increased especially in males with severe OSA and correlated with AHI, ODI and microarousal index as a marker of sleep fragmentation (Xia et al. 2019; Geovanini et al. 2018). Interestingly, only low HDL-cholesterol was associated with OSA in elderly subjects (Roche 2009), in agreement with a low prevalence of the metabolic syndrome in old subjects with OSA (Assoumou et al. 2012) and an overall low profile of OSA severity in advanced age (Monneret et al. 2017). As for the role of sleep structure, the arousal index predicted increased low-density lipoprotein cholesterol (Qian et al. 2016; Martínez-Cerón et al. 2021).

In summary, epidemiologic evidence indicates that metabolic abnormalities constitute a major burden in patients with OSA associated with CIH and sleep fragmentation, with implications regarding the mechanism(s) of increased cardiovascular risk.

3 Studies on Adipose Tissue

Research efforts have tried to dissect the mechanism by which OSA affects metabolism. OSA is frequent in patients with unhealthy obesity, characterized by high cardiometabolic burden and relatively higher expansion of the visceral as opposed to subcutaneous fat (Porro et al. 2021). Experimental data collected during exposure of cells and/or rodents to CIH have been recently summarized (Trzepizur et al. 2018).

Sustained and intermittent hypoxia exert different effects on the adipose tissue. In non-obese mice, chronic exposure to sustained hypoxia for 6 weeks was associated with decreased amount of white adipose tissue (WAT), persistently increased levels of HIF-1α and VEGF expression, and no development of IR in adipocytes (Gozal et al. 2017). Conversely, in non-obese mice exposed to CIH, after an initial increase in HIF-1α transcriptional activity, vascular rarefaction was observed in WAT, together with inflammatory cell infiltration and increased markers of IR in adipocytes (Gozal et al. 2017). These changes were only partially reversible after

interruption of CIH exposure (Gileles-Hillel et al. 2017). There is evidence that CIH causes inflammation of WAT, with activation of macrophages and a shift from the M2 anti-inflammatory to the M1 pro-inflammatory phenotype (Murphy et al. 2017). Dyslipidemic and proatherogenic effects of CIH are mediated by the visceral adipose tissue, since ablation of epididymal fat attenuated the development of vascular lesions in Apo-E deficient mice exposed to CIH (Poulain et al. 2014). Epididymal fat ablation was associated with increased subcutaneous fat deposition and positive effects on metabolic dysfunction (Poulain et al. 2017), indicating a major role of adipose tissue dysfunction and remodeling in CIH-induced alterations. Inflammatory changes in the adipose tissue were more intense in mice with diet-induced obesity than in lean mice (Drager et al. 2011). The effects of CIH, however, partly depend on the experimental model of obesity used. For example, the response to CIH in obese Zucker rats was less severe than in mice with diet-induced obesity (Briançon-Marjollet et al. 2016). Moreover, the effects of CIH may be modulated by the intensity and frequency of hypoxic cycles used in the experiment. Low-frequency intermittent hypoxia may preferentially promote adipogenesis in subcutaneous adipose tissue (Wang et al. 2018).

Adipose tissue hypoxia in obese mice is associated with inflammation (Ye et al. 2007; Hosogai et al. 2007) and is believed to contribute to the metabolic dysfunction of obesity (Engin 2017). Large adipocytes may be exposed to a low pO_2 due to the increased distance between cells and blood capillaries (Trayhurn 2014). Measurements of pO_2 in adipose tissue of rodents showed blunted oscillations of pO_2 levels in vivo during intermittent hypoxia cycles (Almendros et al. 2011), whereas controversial results have been obtained in studies on oxygenation and blood flow in adipose tissue in obese humans (Pasarica et al. 2010; Goossens et al. 2011). A recent study assessed adipose tissue pO_2 in OSA patients and BMI-matched controls. In subcutaneous adipose tissue, pO_2 did not differ between the groups, but OSA patients showed a larger blood flow associated with higher concentrations of inflammatory markers (Thorn et al. 2017). These studies highlight the need for further studies on adipose tissue oxygenation in both obesity and OSA, but the importance of obesity-associated inflammation is firmly established as an intermediate pathogenetic mechanism in both conditions.

Sleep fragmentation in animal models causes a considerable stress response and inflammation, with increased endogenous corticosteroid release and sympathetic activation, partially reversed by uninterrupted sleep (Mishra et al. 2020). Sleep fragmentation in $Apoe^{-/-}$ mice was not associated with altered glucose metabolism, but aggravated atherosclerotic lesions by increasing monocyte production in the bone marrow and decreased release of the wake-promoting orexin-a in the hypothalamus (McAlpine et al. 2019). Interactions between sleep fragmentation, systemic hypertension and gut microbiome composition suggest a very complex picture, still under investigation (Maki et al. 2020). In general, the interaction between sleep fragmentation and metabolism in OSA has been studied much less than other effects, i.e. on blood pressure.

Recent research has investigated molecular mechanisms of CIH-induced insulin resistance in adipose tissue. Caveolae disassembly due to cavin protein

downregulation has been shown in white adipose tissue from lean mice exposed to 6 weeks of CIH, in parallel with development of IR (Varela-Guruceaga et al. 2020). An interesting recent piece of work has linked oxidative stress generated in mitochondria during CIH to a senescence-like phenotype in pre-adipocytes, suggesting that drugs like statins, aspirin, or inhibitors of the renin-angiotensin system might be clinically useful to counteract such changes in obese OSA patients (Polonis et al. 2020). A study on the effects of CIH on cardiovascular gene expression in young and old mice also highlighted the involvement of oxidative stress and cell survival pathways in response to CIH (Castro-Grattoni et al. 2021). Shortened leukocyte telomere length was found in OSA patients in association with short/disturbed sleep (Carroll et al. 2019). The mitochondrial peptide MOTS-C has been identified as a possible early marker of metabolic dysfunction in obese OSA patients, since its serum levels were negatively correlated with both AHI and BMI (Baylan and Yarar 2021). Other studies have explored the cross-talk between macrophages and circulating exosomes in OSA-associated metabolic dysfunction (Khalyfa et al. 2018a) and the role of circulating exosomes in modulating insulin sensitivity in adipocytes (Khalyfa et al. 2018b). Very sophisticated techniques have revealed functional heterogeneity in the response of white adipose tissue to intermittent hypoxia at the single-cell level, with differential gene activation in different cell types (Khalyfa et al. 2021a). CIH modifies gut microbiota, increases epithelial permeability, and modifies plasma exosome cargo; these alterations are associated with development of IR (Khalyfa et al. 2021b). Sleep fragmentation also modifies gut microbiota and is associated with altered feeding behavior, IR, and adipose tissue inflammation (Poroyko et al. 2016). The current state of the art on metabolomics and microbiome in OSA has been recently summarized (Zhang et al. 2021).

In summary, OSA represents a highly complex and interesting model which opened the way to the studies on the effects of chronic intermittent hypoxia on adipose tissue pathophysiology in obesity. Animal and cell models will continue to be essential to unravel the independent effects of OSA and obesity on adipose tissue and cardiovascular risk.

4 Effects of CPAP Treatment

Many clinical studies in OSA patients assessed the effects of CPAP treatment on metabolic variables. The majority of these studies have been negative, as summarized in meta-analyses, with the exception of a positive effect of CPAP treatment on insulin resistance in both nondiabetic (Abud et al. 2019) and diabetic patients (Chen et al. 2014). HbA1c and fasting blood glucose were unchanged after CPAP treatment (Labarca et al. 2018; Zhu et al. 2018). CPAP treatment was not associated with changes in the amount of subcutaneous (Chen et al. 2020) or visceral adipose tissue (Iftikhar et al. 2015). Two meta-analyses found decreased leptin levels after CPAP treatment (Zhang et al. 2014; Chen et al. 2015a), while adiponectin levels were unaffected (Chen et al. 2015b). Lipid levels showed decreased total and LDL cholesterol, and increased HDL-cholesterol after OSA treatment (Nadeem et al.

2014). These results have to be critically considered for several reasons. First, the number of patients examined in the cited meta-analyses was small, i.e. on average < 500 patients, indicating the need for further assessment in larger samples. Second, compliance to CPAP treatment by OSA patients is highly variable, with many patients using CPAP for <4 h/night. Therefore, negative results could also reflect insufficient CPAP treatment. A good example is a proof-of-concept randomized controlled trial in which patients with OSA and prediabetes slept in the laboratory and used CPAP for 8 h/night for 2 weeks. The area under the curve of the oral glucose tolerance test was significantly lower in CPAP-treated patients than in placebo-treated controls after short-term CPAP use (Pamidi et al. 2015). In a similar study in diabetic OSA patients, 24-h glucose improved after just 1 week of CPAP treatment (Mokhlesi et al. 2016). Both these studies suggest that an improvement in metabolic variables might be observed only in patients highly compliant to CPAP treatment. REM sleep predominates in the second part of the night, and full-night CPAP use would prevent the highly desaturating respiratory events in REM sleep, which are associated with metabolic changes and sympathetic activation (Grimaldi et al. 2014; Varga and Mokhlesi 2019).

Other interesting data regard the comparison of the effects of weight loss, alone or in combination with CPAP treatment, vs CPAP treatment alone for 24 weeks, on metabolic variables. C-reactive protein, IR, and triglyceride levels decreased only in the groups undergoing weight loss, while they were unchanged in the CPAP-only treatment group (Chirinos et al. 2014). Therefore, weight loss exerts powerful effects on metabolism, and an effect of CPAP on metabolic variables can only be expected when CPAP treatment is associated with hypocaloric diet.

5 New Drugs to Treat Obesity: Effects in OSA Patients

The importance of addressing obesity besides treating OSA is increasingly recognized, since CPAP treatment has a little impact on metabolic variables. The low level of CPAP acceptance by some clinical OSA phenotypes (Bailly 2021) increased the interest in new pharmacologic approaches to treat either OSA, or obesity, or both. Some drugs, initially developed for type 2 diabetes, are increasingly used to treat obesity and its complications (Papaetis 2021). They belong to 2 main classes: glucagon-like peptide 1 (GLP-1) analogues and sodium glucose cotransporter 2 (SGLT2) inhibitors. They could be useful in OSA patients, especially to help reduce body weight and cardiovascular risk in severely obese subjects (Brown et al. 2021).

One expected result of weight loss is a decrease in OSA severity, since a linear relationship exists between AHI and BMI (Peppard et al. 2000). Consistent with this relationship, decreased OSA severity was documented in small studies in obese patients with type 2 diabetes after treatment with a SGLT2 inhibitor (Furukawa et al. 2018; Sawada et al. 2018; Tang et al. 2019). The recent post-hoc analysis of the EMPA-REG OUTCOME trial in patients with type 2 diabetes reported a higher prevalence of obesity and incidence of cardiovascular and renal outcomes in patients

with OSA compared to those without OSA at study entry (Neeland 2020). Empagliflozin exerted strong protective effects on cardiovascular and renal outcomes independent of the OSA status; a trend toward larger weight loss in OSA than non-OSA patients might explain the lower incidence of OSA in empagliflozin-treated patients compared to the placebo group. The study did not include data on OSA severity or OSA treatment (Neeland 2020). A meta-analysis confirmed that SGLT2 inhibitors decreased the risk for OSA (Qiu et al. 2021). Similarly promising results have been reported for use of the GLP-1 agonist liraglutide in obese OSA patients (Blackman et al. 2016). A study on diabetic obese patients reported decreased daytime sleepiness after liraglutide treatment, but sleep studies were not obtained, making it impossible to evaluate whether decreased OSA severity might explain this result (Gomez-Peralta et al. 2015). A randomized multicenter clinical trial is ongoing, to assess the effects of liraglutide, alone or combined with CPAP treatment, on OSA severity and metabolic variables in diabetic OSA patients compared to CPAP treatment alone (Sprung et al. 2020). These drugs open new perspective in the treatment of obese OSA patients, and this is especially relevant given the low acceptance of CPAP treatment in many OSA patients, especially in the long term.

6 Conclusions

OSA and obesity frequently coexist and may worsen adipose tissue metabolism through common pathogenetic mechanisms. The data discussed so far indicate that a clear evidence is available only for an independent effect of OSA on insulin resistance, for which positive effects of OSA treatment have also been shown. Figure 1 summarizes our current state of knowledge on the OSA-obesity interactions. The available data suggest that OSA contribute to worsen the metabolic abnormalities of obesity. Lack of exercise increases the risk to develop both OSA and obesity. Therefore, the best clinical strategy in OSA patients remains OSA treatment associated with lifestyle interventions against obesity and lack of physical exercise, given their strong power in decreasing cardiometabolic risk.

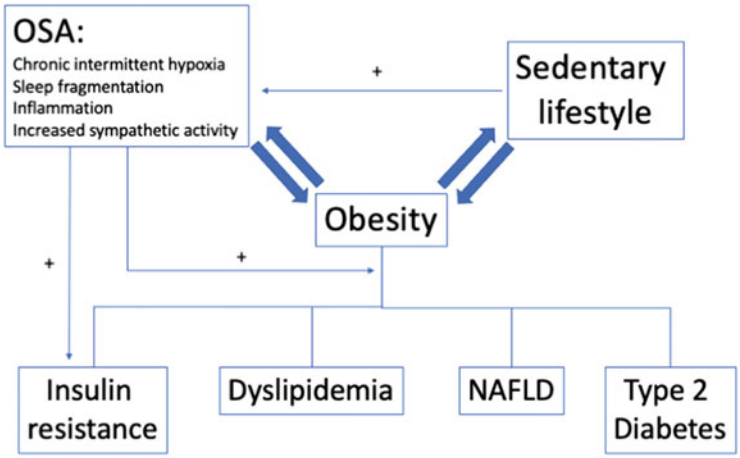

Fig. 1 Summary of the interaction between OSA and obesity, and their health consequences. OSA causes insulin resistance independent of obesity, and may contribute to worsen obesity-related metabolic diseases such as type 2 diabetes, dyslipidemia, and non-alcoholic fatty liver disease (NAFLD). Increased physical activity and hypocaloric diet should always be indicated in OSA patients, in adjunct to CPAP treatment

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Part II

Diabetes and Complications

Inter-Organ Crosstalk in the Development of Obesity-Associated Insulin Resistance

Megan Piquet, M. Carmen Martínez, and Tania Romacho

Contents

Abstract

The epidemics of obesity and type 2 diabetes have led to intensive investigation of the underlying mechanisms of these diseases and their main complications such as cardiovascular diseases and non-alcoholic fatty liver disease. This search has contributed to better understand how organs and tissues communicate with each other in the so-called inter-organ crosstalk. Adipose tissue, the liver, or skeletal muscle can actively release secreted factors termed "organokines" which

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can interact with other distant targets in complex networks. More recently, other novel mediators of inter-organ crosstalk such as extracellular vesicles and their non-traditional cargoes as miRNAs and lncRNAs are gaining importance and represent potential therapeutic targets. In the present chapter we summarize some of the current knowledge on inter-organ communication with a focus on adipose tissue-released factors and their modulation on other organs and tissues like pancreas, liver, skeletal muscle, the cardiovascular system, and the gut in the context of obesity and its progression to insulin resistance. We also provide a perspective on mediators of inter-organ crosstalk as potential therapeutic targets.

Keywords

Crosstalk · Organokines · Insulin resistance · Obesity · Type 2 diabetes

Abbreviations

AMPK	AMP-activated protein kinase
ASC	Adipose-derived stem cell
AT	Adipose tissue
ATPase 2a	Adenosine triphosphatase 2a
BMI	Body mass index
DPP4	Dipeptidyl peptidase-4
eNampt	Visfatin/nicotinamide phosphoribosyltransferase
eNOS	Endothelial nitric oxide synthase
ERK1/2	Extracellular signal-regulated kinases 1/2
EVs	Extracellular vesicles
FABP4	Fatty acid-binding protein 4
FFA	Free fatty acid
FGF21	Fibroblast growth factor 21
FOXO	Forkhead box protein O
GSK-3β	Glycogen synthase kinase 3 beta
HUVEC	Human umbilical vein endothelial cells
IFNγ	Interferon-γ
IL-1β	Interleukin 1β
IL-6	Interleukin
LncRNAs	Long non-coding ribonucleic acids
LPS	Lipopolysaccharide
MCP-1	Monocyte chemoattractant protein 1
M-CSF	Macrophage colony-stimulating factor
miRNA	Micro ribonucleic acid
mRNA	Messenger ribonucleic acid
mTOR	Mechanistic target of rapamycin
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NF-κB	Nuclear factor-kappa B

NO	Nitric oxide
PI3-K	Phosphatidylinositol 3-kinase
PPARα	Peroxisome proliferator-activated receptor alpha
ROS	Reactive oxygen species
SCFA	Short-chain fatty acids
sDPP4	Soluble DPP4
SFA	Saturated fatty acids
SVF	Stromal vascular fraction
T2D	Type 2 diabetes
TGF-β1	Transforming growth factor β1
TLR4	Toll-like receptor 4
TNFα	Tumor necrosis factor α
TSC1	Tuberous sclerosis complex 1
VEGF	Vascular endothelial growth factor
VSMCs	Vascular smooth muscle cells

1 From Obesity to Diabetes: The Role of Inter-Organ Crosstalk

Obesity is characterized by an excess of fat that can lead to a deleterious effect on health (Carraro et al. 2021). According to the WHO, more than 650 million people worldwide are clinically obese, and this prevalence continues growing (WHO 2016). The acquisition of a Western diet and lack of exercise have extensively contributed to the obesity epidemics. Besides a "thermodynamic imbalance" between caloric intake and energy expenditure, obesity is a very complex disease influenced as well by genetics, epigenetics, and psychosocial factors and therefore effective therapies are urgently needed (Carraro et al. 2021). Obesity, and especially abdominal obesity, is often accompanied by other metabolic disturbances such as enhanced fasting plasma glucose, hypertension, and dyslipidemia. These factors frequently cluster together in the metabolic syndrome (Alberti et al. 2009). Metabolic syndrome increases the risk to develop type 2 diabetes (T2D) and cardiovascular diseases 2–4 times (Ballantyne et al. 2008). In fact, in T2D patients, the accumulation of visceral fat represents a key promoter of atherosclerosis, even independently of other traditional risk factors (Reijrink et al. 2019). While subcutaneous fat can act as a "metabolic sink," visceral fat is specially related to the cardiometabolic complications of obesity. Metabolic disturbances such as obesity and insulin resistance lead to a pro-inflammatory state in white adipose tissue (AT) which reduces adipose-derived stem cells (ASCs) plasticity leading to AT remodeling mostly through hypertrophy (Badimon and Cubedo 2017). Furthermore, visceral fat has a more reduced ability to store free fatty acids (FFAs) than subcutaneous fat and displays a different lipidomic signature in overweight humans (Zacharia et al. 2020). Visceral AT is more prone to hypertrophic remodeling and macrophage recruitment.

Due to its location, visceral fat can release FFAs and other soluble factors into systemic circulation contributing to chronic inflammation and leading to lipid overflow resulting in ectopic fat accumulation in skeletal muscle and the liver and, eventually, causing insulin resistance (Després and Lemieux 2006). The elevation of fasting and postprandial plasma glucose levels is the outcome of pancreatic β-cell failure after a long period compensating insulin resistance in peripheral tissues (Kahn et al. 2006). Although it is clear how this process ends, the underlying mechanisms of how insulin resistance begins are very complex and remain yet not fully understood. Diabetes is defined by enhanced plasma levels of a single metabolite, glucose, but there is emerging evidence that inflammation also plays a role in T2D progression and its further complications. Obesity and T2D are considered chronic subclinical inflammatory states. This persistent, subacute inflammation is acknowledged as a key factor promoting metabolic derangements and finally contributing to insulin resistance in obesity. Under these conditions, excessive adipocyte growth leads to AT dysfunction characterized by a shift of the AT secretome with increased pro-inflammatory M1 macrophages but also an increased release of pro-inflammatory factors by adipocytes themselves (Romacho et al. 2014). Indeed, AT dysfunction has a central role in the development of insulin resistance, T2D, and key complications such as cardiovascular diseases and non-alcoholic fatty liver disease (NAFLD).

Insulin resistance in the AT results in reduced inhibition of lipolysis and increased lipogenesis. Besides triglyceride storage, AT acts as a true endocrine organ which synthesizes and releases a wide range of mediators (Romacho et al. 2014). Other circulating metabolic mediators such as lipids, amino acids, and keto acids also play an important role in inter-organ crosstalk regarding the onset of insulin resistance (Gancheva et al. 2018). Nevertheless, we will focus on AT-derived factors and how they can regulate distant targets, such as the liver, skeletal muscle, pancreas, the cardiovascular system, and gut microbiota. This chapter aims to provide an overview on relevant mediators of inter-organ crosstalk promoting or preventing the progression from obesity to insulin resistance. The impact of soluble molecules such as organokines, circulating soluble lipid mediators such as FFAs and extracellular vesicles (EVs) together with their most relevant/promising cargos in insulin resistance pathogenesis will be revised. Finally, we provide a perspective on potential therapeutic targets to interfere in this complex network in the context of metabolic diseases.

2 Mediators of Communication: Soluble Factors of Inter-Organ Crosstalk

In both health and insulin resistance states, AT, skeletal muscle, and the liver act as important endocrine organs (Fig. 1). Therefore, among the soluble inter-organ crosstalk mediators, adipokines, myokines, and hepatokines are critical actors. These soluble factors are very heterogeneous and comprise classical cytokines like interleukin(IL)-1β, IL-6, prototypical molecules specifically originated in the AT

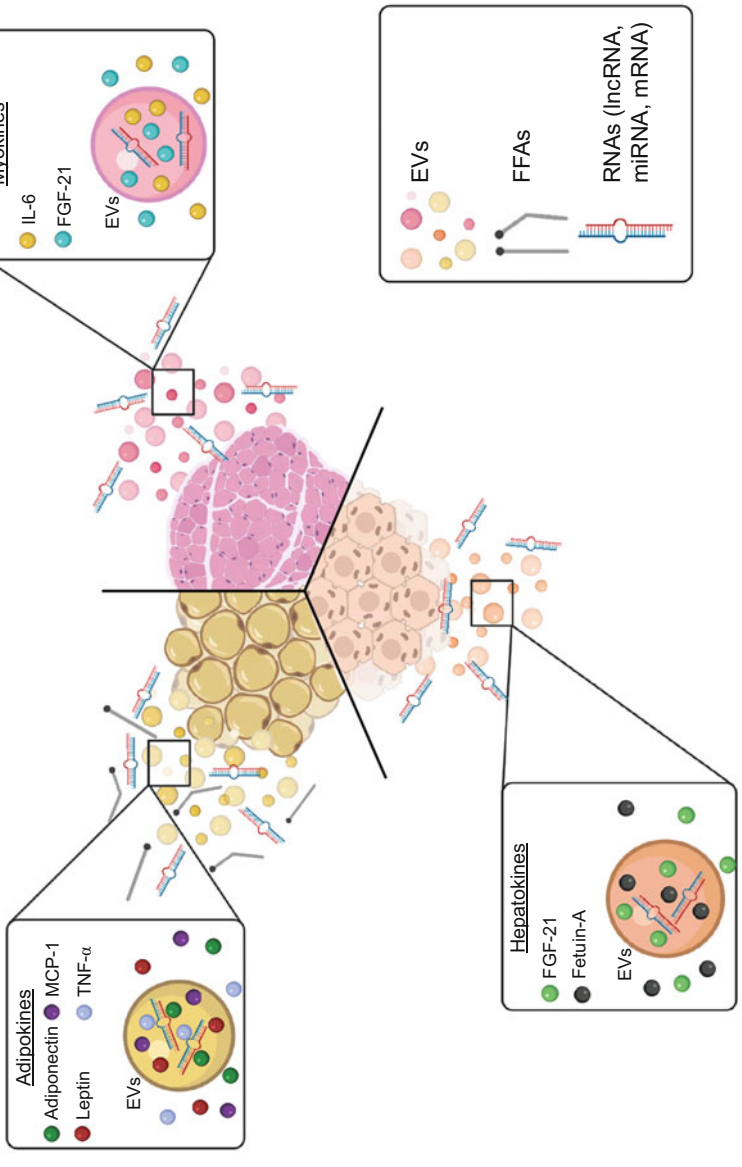

Fig. 1 Mediators of inter-organ communication released by different tissues. The main soluble cytokines are represented for each tissue as well as the free fatty acids and the circulating or internalized RNAs into extracellular vesicles. *EVs* Extracellular vesicles, *FFAs* Free fatty acids, *FGF21* Fibroblast growth factor 21, *IL-6* Interleukin-6, *LncRNAs* Long non-coding ribonucleic acids, *MCP-1* Monocyte chemoattractant protein 1, *miRNA* Micro ribonucleic acid, *mRNA* Messenger ribonucleic acid, *TNF-α* Tumor necrosis factor-α

like adiponectin or leptin, or in the skeletal muscle as myostatin, or simultaneously by AT, muscle, and liver as fibroblast growth factor 21 (FGF21) (Itoh 2014). Under pathophysiological conditions as obesity or T2D, there is an imbalance in these factors with a prominent production of pro-inflammatory factors (Oh et al. 2016). However, although extensive evidence has demonstrated an improvement of insulin signaling under lifestyle modifications such as diet or exercise, it remains elusive how these factors interplay with each other and what therapeutic potential they hold to combat the metabolic syndrome.

2.1 Adipokines

It is worth highlighting that AT comprises besides mature adipocytes and other non-parenchymal cell types, the so-called stromal vascular fraction (SVF). The SVF contains vascular cells, preadipocytes, fibroblasts, immune and multipotent stem cells. The term adipokine initially referred to a protein produced and released from adipocytes (Trayhurn and Wood 2004), thus excluding factors from other cells within the AT, but this term is currently used indistinctly for factors derived from all AT components. Adipokines can in turn be directly released by adipocytes through different mechanisms (Romacho et al. 2014) but also embedded in EVs (Hartwig et al. 2019). Thus, the whole fat cell secretome comprises the adipokinome, the novel term "exoadipokinome" (Hartwig et al. 2019) plus other lipid species such as FFAs or ceramides (Dahlman et al. 2012).

The discovery of classical adipokines like leptin, which has a key role in appetite control, and the insulin-sensitizing and pleiotropic anti-inflammatory adipokine adiponectin (Wang and Scherer 2016) has led to acknowledge the importance of AT in inter-organ crosstalk and specially in the context of the pathogenesis in the progression from obesity, insulin resistance, and T2D (Romacho et al. 2014). Besides prototypical AT-derived classical cytokines with prominent roles in the pathophysiology of diabetes and its complications such as IL-1β or tumor necrosis factor α (TNFα), there are other factors not restricted to the AT such as the components of the renin-angiotensin system. Besides markers of systemic inflammation and cardiometabolic risks, adipokines are acknowledged as real mediators in defective inter-organ crosstalk leading to insulin resistance as well as promoting complications of T2D as cardiovascular diseases.

2.2 Myokines and Hepatokines: Toward the Organokine Concept

Skeletal muscle and liver can also act as endocrine organs through the release of factors called myokines and hepatokines, respectively. The term "organokine" collectively refers to adipokines, myokines, hepatokines, and other -kines to come (Choi 2016). Myokines are defined as cytokines and other kind of peptides synthesized, expressed, and secreted by muscle fibers which can act in an auto-/para- or endocrine manner (Pedersen and Febbraio 2012). However, many myokines

are not solely produced by skeletal muscle but also by AT such as IL-6 or also by the liver, like FGF21. Insulin resistance in skeletal muscle is a key step in the initiation of T2D. Thus, in insulin resistant skeletal muscle, intramyocellular lipids are accumulated and the myokinome profile is modified. Furthermore, upon sedentarism or exercise, adipokines and myokines can interact and regulate each other. In line, it has been proposed that myokines are the mediators of the beneficial effects of exercise.

Hepatokines such as FGF21 and fetuin-A are proteins produced and released mainly by the liver and can regulate glucose and lipid homeostasis in a positive or negative manner. Similarly to the previous "organokines," diet, metabolic status, and exercise can modulate these factors. Thus, acute high-fat overfeeding increases circulating levels of FGF21 and fetuin-A in healthy men (Willis et al. 2020). Acute exercise also increases circulating FGF21 in an intensity-dependent manner (Willis et al. 2019).

2.3 EVs as Shuttle of Biological Information

Due to their cargo, EVs can be considered as true messengers between organs. Indeed, EVs are released from "mother cells" through different mechanisms and transfer their content to other cells (recipient or target cells). EVs are membrane structures released from the blebbing of the plasma membrane or from intracellular components such as intraluminal vesicles and multivesicular bodies. Depending on their size, two subtypes can be distinguished: large EVs and small EVs. During their biogenesis, EVs are enriched in proteins, lipids, messenger ribonucleic acid (mRNA), microRNAs (miRNA), and other types of RNA as well as in fragments of organelles. Due to their different biogenesis mechanisms, large EVs and small EVs are enriched in different proteins allowing their characterization. Indeed, large EVs are rich in plasma membrane markers that allow determining the cell of origin. Thus, it has been reported that large EVs can be generated from platelets, leukocytes, erythrocytes, hepatic cells, tumor cells and, in general, from all types of cells. At difference of large EVs, small EVs are rich in tetraspanin proteins showing that the machinery involved in the EV biogenesis determines the EV content. However, common components of both types of EVs have been also described. Hence, recent works show that both types of EVs can carry molecules that up to now have been considered as "soluble." For instance, large EVs released from lipopolysaccharide (LPS)-treated monocytes harbor bioactive IL-1β and transcripts for pro-inflammatory cytokines such as TNFα, IL-6, and IL-8 (Mackenzie et al. 2001; Wen et al. 2014). Circulating EVs from non-obese and obese patients carry several cytokines that correlated with body mass index (BMI) showing a link between the content of EVs and obesity although no evidence of the functionality of these cytokines has been demonstrated in vivo (Amosse et al. 2018). Also, circulating large EVs from mice contain chemokines and cytokines such as monocyte chemoattractant protein 1 (MCP-1), macrophage colony-stimulating factor

(M-CSF), and IL-1β and induce pro-inflammatory response in endothelial cells (Gaceb et al. 2016).

Concerning the lipid content, several studies have shown an increase in cholesterol, sphingomyelin, and phosphatidylserine in EVs when compared to mother cells, whereas phosphatidylcholine and phosphatidylinositol contents were decreased (for review, see Skotland et al. 2020). But as indicated above for cytokines, this is not clear whether changes in the composition of lipid EVs can modify their biological actions. Recently, it has been reported that ceramide C16:0 generated in thoracic AT from the paracardial region is secreted in small EVs released from the adipocytes (Akawi et al. 2021). Finally, EVs are very rich in mRNA and miRNA. Initial reports indicate that the lipid envelope of EVs protect mRNA and miRNA from degradation in comparison with circulating mRNA and miRNA (Kosaka et al. 2010). Thus, EVs can transfer both types of RNA to long-range target cells and induce changes on function of target cells. All these effects induced by the cargo of EVs will be further developed below.

2.4 Other Circulating Messengers (miRNA, lncRNA, Fatty Acids)

miRNA are a class of non-coding RNAs with less than 20 to 24 nucleotides (Ling et al. 2013). Unlike other types of RNAs, miRNAs regulate the expression of certain genes by base-pairing to the 3′UTR of their target mRNA. They are produced by two RNase III proteins, Drosha and Dicer, and generally target mRNAs from their cell of origin. Although miRNA levels in blood are relatively stable when circulating free in blood, it has been shown that miRNAs can be transported and protect from degradation into EVs or by binding to lipoprotein complexes (Chen et al. 2008; Kosaka et al. 2010).

Long non-coding RNAs (LncRNAs) are transcripts of more than 200 nucleotides that compose the majority of the nonprotein-coding genome. They regulate genes at the transcriptional and epigenetic level by direct interaction with chromatin or different transcription factors. Like miRNAs, LncRNAs can be found within EVs and act to target cells and tissues at long-range (Viereck and Thum 2017). These encapsulated circulating LncRNAs are stable in the extracellular environment, resisting to temperature or pH changes or to RNase attack, although they are preserved from degradation when carried by EVs. Moreover, it has been shown that in several pathologies such as obesity, circulating LncRNAs are enriched or decreased in the bloodstream, making them excellent biomarkers. For example, Thomou et al. (2017) have demonstrated the importance of miRNAs carried by small EVs derived from AT in the pathogenesis of metabolic dysfunctions.

Finally, during obesity, levels of plasma FFAs released from AT expansion, but also those consumed through diet, are increased. FFAs are classified according to the absence, saturated fatty acids (SFAs), or presence of double bonds (monounsaturated or polyunsaturated fatty acids, MUFAs or PUFAs, respectively) in their structure. Whereas SFAs are described as inducing deleterious effects such as insulin resistance and inflammation in a large variety of tissues (Boden et al. 2005),

interestingly, gut microbiota produces short-chain fatty acids (SCFA) including acetate, propionate, and butyrate that can modulate different metabolic pathways related with obesity, insulin resistance, and T2D. In general, SCFA possess anti-inflammatory effects and the dysbiosis associated with a higher fat and decrease of dietary fibers consumptions described during obesity and diabetes disrupts the SCFA generation leading to an increase in permeability and inflammation of the intestinal epithelium (Yehualashet and Yikna 2021).

3 Organ Crosstalk

3.1 AT-Liver Crosstalk

Secreted adipokines such as adiponectin, IL-6, leptin, MCP-1, and FFAs from AT play a critical role in the development of multi-organ insulin resistance including the liver. In detail, visceral AT removal improves hepatic insulin resistance in diet-induced obese rats (Ben-Shlomo et al. 2012). Recently, it has been shown that removal of epididymal visceral AT enhances insulin-stimulated Akt phosphorylation, not only in liver, but also in skeletal muscle. Interestingly, this was associated with a decrease of hepatic steatosis and oxidative stress, an increase in circulating adiponectin levels and a decrease in IL-6 and FFAs (Franczyk et al. 2021).

Since adiponectin exerts hepatoprotective actions, reduced adiponectin levels and decreased hepatic expression of the two adiponectin receptors during obesity are related to the severity of steatosis, inflammation, and fibrosis (Balmer et al. 2010) and contribute to a state of hepatic adiponectin resistance (Combs and Marliss 2014). The molecular mechanisms involve the specific interaction of adiponectin with its receptor AdipoR2, and the subsequent activation of both AMP-activated protein kinase (AMPK) downstream signaling and peroxisome proliferator-activated receptor alpha (PPARα) in the liver attenuating hepatic lipoinflammation (Ishtiaq et al. 2019).

During obesity, enhanced secretion of several adipokines such as MCP-1 can exacerbate the infiltration of macrophages in the AT, systemic insulin resistance, and increased hepatic triglyceride content in mice (Kanda et al. 2006). On the contrary, deletion of MCP-1 gene or inhibition of MCP-1 improved insulin resistance and lipid ectopic accumulation in the liver of obese mice (Kanda et al. 2006).

Dipeptidyl peptidase- 4 (DPP4) can cleave and inactivate the incretin hormones among other factors (Röhrborn et al. 2015). We have shown that soluble DPP4 (sDPP4) is an adipokine predominantly secreted by visceral AT in humans (Sell et al. 2013). Others propose that it is mainly produced by the liver. Regardless of its main source, sDPP4 promotes insulin resistance and inflammation in vitro and in vivo (Ghorpade et al. 2018). Thus, specific DPP4 deletion in the AT improves hepatic insulin sensitivity in a murine model of diet-induced obesity (Romacho et al. 2020a, b), whereas DPP4 silencing in hepatocytes suppresses the secretion of pro-inflammatory adipokines such as MCP-1, IL-6, TNFα, and IL-1β from visceral AT and insulin resistance (Ghorpade et al. 2018; Baumeier et al. 2017). However,

orally administered DPP4 inhibitors, like sitagliptin, do not exert similar effects as DPP4 silencing in liver or AT (Ghorpade et al. 2018).

Other strategies such as inhibition or deletion in the liver of proteins involved in the SUMOylation (Liu et al. 2021), Forkhead box protein O (FOXO) transcription factors (Garcia Whitlock et al. 2021) and hepatokines, mainly FGF21, have shown a reduction of AT mass and decreased insulin resistance suggesting that the crosstalk between both organs is bidirectional.

3.2 AT-Skeletal Muscle Crosstalk

AT and skeletal muscle are the largest endocrine organs in humans. The upregulation of certain pro-inflammatory adipokines during obesity can affect skeletal muscle insulin sensitivity. It has been proposed that adipokines promote lipotoxicity, even in early stages of obesity when plasma FFA levels are not yet influenced by lipolysis. Thus, conditioned medium from human adipocytes in combination with low concentrations of palmitate potentiated lipid accumulation, reduced palmitate oxidation, and increased diacylglycerol content in human skeletal muscle cells (Taube et al. 2012).

On the contrary, the myokine secretory profile can be positively modulated by exercised-induced muscle contraction releasing the so-called exercise factors that comprise not only myokines, but also other metabolites which can counteract the negative effects of obesity-related adipokines or cytokines. In line, contraction of human skeletal muscle cells by electrical pulse stimulation activated AMPK activation and induced IL-6, similarly to what described for exercise in vivo. Furthermore, electrical pulse stimulation blunted insulin resistance induced by adipocyte conditioned medium, or isolated factors as MCP-1 and chemerin, by blocking pro-inflammatory signaling pathways (Lambernd et al. 2012). In spite of the identification of many novel myokines, their real impact in inter-organ crosstalk in humans remains unclear (Eckel 2019). Very recently, the regulation of EVs released from limbs under muscle contraction to the circulation and its uptake has demonstrated that EVs participate in exercise-mediated inter-organ crosstalk (Whitham et al. 2018).

3.3 AT-Pancreas Crosstalk

It is well accepted that adipokines and other mediators released by AT play a major role in the development of insulin resistance by acting on both peripheral organs and on pancreas function. Increased circulating fatty acid-binding protein 4 (FABP4) levels are associated with blunted increased insulin secretion, insulin sensitivity, and elevated glucagon fasting and oral glucose postchallenge in patients with T2D (Wang et al. 2021a) suggesting that FABP4 may affect β-cell and α-cell function. Both adiponectin and leptin levels act in an endocrine manner to regulate β-cell function since adiponectin increases and leptin inhibits insulin production (Turer and

Scherer 2012; Iikuni et al. 2008). Indeed, in obesity, increased leptin and decreased adiponectin levels reduce glucose-induced insulin secretion leading to β-cell secretory insufficiency. Other adipokines such as chemerin, adipsin, and apelin, through the overexpression of G protein-coupled receptors, can also stimulate insulin secretion (Atanes et al. 2021).

Lipotoxicity induced by FFAs impairs pancreatic β-cell function resulting in a decrease in glucose-stimulated insulin secretion (Maedler et al. 2003). More in detail, ceramides induce β-cell apoptosis by releasing cytochrome c from the mitochondria (Lang et al. 2011) whereas palmitate inhibits insulin gene expression by acting at the transcriptional level via de novo ceramide synthesis (Kelpe et al. 2003). On the contrary, palmitic acid esters of hydroxy stearic acid released from AT are considered as endogenous anti-diabetic and anti-inflammatory lipids since they enhance glucose-stimulated insulin secretion and β-cell survival in pancreas (Syed et al. 2019).

Very recently, it has been shown that EVs are involved in a functional crosstalk between AT and pancreatic β-cells, which positively or negatively influences β-cell fates, depending on the physiopathological state of adipocytes and AT of origin (Gesmundo et al. 2021). Thus, EVs from untreated adipocytes induce cell survival, proliferation, AKT, and glycogen synthase kinase 3 beta (GSK-3β) phosphorylation and reduce apoptosis of pancreatic β-cells. Whereas EVs released from cytokine-treated adipocytes reduce cell survival, proliferation, AKT and GSK-3β phosphorylation and increase β-cell apoptosis. Although the underlying mechanisms mediating these effects are not completely elucidated, differential miRNA expression between both types of EVs may explain, at least partly, these effects.

3.4 Interaction Between AT and the Cardiovascular System

As described above, during obesity, changes in the AT secretome induce metabolic dysfunctions and inflammation including alteration of cardiovascular homeostasis (Chait and Den Hartigh 2020). Cardiovascular dysfunction, whose early hallmark is endothelial dysfunction, is associated with an imbalance in the expression and secretion of pro-angiogenic, pro-atherothrombotic, pro-inflammatory factors by AT (Wronkowitz et al. 2014). Consequently, identifying the detailed crosstalk between adipocytes and cardiovascular cells may help to prevent obesity-associated cardiovascular diseases (Wronkowitz et al. 2014).

During obesity, the rapid expansion of AT affects tissue vascularization resulting to a decreased oxygen supply in adipocytes, which leads to hypoxia, inflammation, and deficient angiogenesis (Hodson et al. 2013). During adipocyte differentiation, the expression of vascular endothelial growth factor (VEGF) secreted by AT is increased leading to vascular smooth muscle cells (VSMCs) proliferation of in vitro (Schlich et al. 2013), increased angiogenesis and insulin sensitivity within AT (Elias et al. 2012).

Paradoxically, in obesity and insulin resistance, central leptin resistance is accompanied by hyperleptinemia which has been associated with hypertension,

acute myocardial infarction, and heart failure (Fujimaki et al. 2001; Schulze et al. 2011; Simonds et al. 2014). This can be due to leptin ability to stimulate sympathetic nerve activity (Simonds et al. 2014) through the phosphatidylinositol 3-kinase (PI3-K) and extracellular signal-regulated kinases 1/2 (ERK1/2) pathways (Rahmouni et al. 2009). Similar effects have been described for resistin. Indeed, plasma levels of resistin have been positively correlated with high blood pressure. The involved mechanism seems to be related with the enhancement of renal sympathetic nerve activity via PI3-K (Kosari et al. 2012). Interestingly, an additive effect of leptin and resistin has been described when both adipokines were co-administrated in rats (Habeeballah et al. 2016). In in vitro studies using endothelial cells, leptin increases reactive oxygen species (ROS) production leading to fatty acid oxidation and reduced nitric oxide (NO) bioavailability through the endothelial nitric oxide synthase (eNOS) uncoupling promoting endothelial dysfunction (Korda et al. 2008).

We have described that visfatin/Nicotinamide phosphoribosyltransferase (eNampt), a multi-faceted adipokine whose circulating levels are enhanced in T2D and obesity, causes endothelial dysfunction in vivo and ex vivo as well as vascular inflammation (Romacho et al. 2020a; Vallejo et al. 2011). Interestingly, in vivo endothelial dysfunction evoked by visfatin infusion was mediated by NOD-like receptor family, pyrin domain containing 3 (NLRP3)-inflammasome-driven tissular release of IL-1β (Romacho et al. 2020b).

Since adiponectin exerts protective effects on vascular endothelial cells, VSMCs, and cardiomyocytes, the decrease in adiponectin levels observed during obesity is associated to poorer outcomes in cardiovascular diseases (for review, see Nakamura et al. 2014). In detail, adiponectin downregulation decreases eNOS activity leading to decreased NO bioavailability. Also, adiponectin favors prostacyclin production (Ohashi et al. 2009) and decreases secretion of pro-inflammatory cytokines from endothelial cells by inhibiting nuclear factor-kappa B (NF-κB) signaling (Ouchi et al. 2000).

Omentin, an anti-inflammatory adipokine produced by AT, mainly from the SVF, that is downregulated during obesity, improved endothelium dysfunction by decreasing ROS production and increasing NO bioavailability in obese diabetic rats (Leandro et al. 2021). Recently, omentin-1 levels have been reported to be decreased in patients with hypertension compared with normotensive controls (Çelik et al. 2021) and atrial fibrillation (Chen et al. 2020). Interestingly, omentin displayed anti-fibrotic actions by counteracting transforming growth factor-(TGF)-β1-induced cardiac fibroblast activation and endothelial-to-mesenchymal transition (Chen et al. 2020). Under high glucose conditions, omentin protects against NO decrease and ROS production in endothelial cells via the AMPK/PPARδ signaling pathway (Liu et al. 2020). Conversely, recent data show that elevated levels of circulating omentin are associated with an increased risk of stroke and cardiovascular death in individuals with diabetes after adjustment for multiple cardiovascular risk factors (Niersmann et al. 2020) suggesting a potential role of omentin in the development of cardiovascular diseases.

Of particular interest, perivascular AT, the fat depot surrounding vessels, through the release of adipokines, FFAs, chemokines, and relaxing and contracting factors,

plays a key role in the crosstalk between the AT and the cardiovascular system due to its privileged localization. Accumulation of perivascular AT during obesity and diabetes decreases the release of perivascular adipose relaxing factors and exacerbates the production of perivascular contracting factors resulting in vasoconstriction (Britton and Fox 2011). For instance, reduced adiponectin expression in perivascular AT in T2D patients was increased and positively correlated with vascular oxidative stress (Antonopoulos et al. 2015). Conditioned medium from epicardial AT of patients with T2D reduced sarcomere shortening, cytosolic calcium fluxes, expression of sarcoplasmic endoplasmic reticulum adenosine triphosphatase 2a (ATPase 2a), and decreased insulin-mediated AKT-Ser473-phosphorylation of isolated rat cardiomyocytes. These effects were due to the enrichment in activin A and angiopoietin-2 in conditioned medium (Greulich et al. 2012).

As indicated above, EVs can be involved in the dialogue between AT and the cardiovascular system. A transfer of caveolin-1 from glucagon-stimulated neighboring endothelial cells to adipocytes takes place in mice in vivo (Crewe et al. 2018). Delivery of miR-200a from adipocyte-derived EVs to cardiomyocytes resulted in decreased tuberous sclerosis complex 1 (TSC1) and subsequent mechanistic target of rapamycin (mTOR) activation, leading to cardiomyocyte hypertrophy in vitro (Fang et al. 2016). On the other hand, EVs from adipose-derived stromal cells displayed cardioprotective effects in acute myocardial infarction in rats. Hence, under hypoxic conditions, these EVs are enriched in miR-93-5p that can be delivered to cardiomyocytes. As a result, Atg7 and Toll-like receptor 4 (TLR4)/NF-κB were downregulated, leading to suppressing cardiac injury-mediated autophagy and inflammatory responses, respectively (Liu et al. 2018).

In this regard, LPS found in blood has been described as associated to sEVs from patients with metabolic syndrome and via the activation of TLR4 induces endothelial dysfunction through the overproduction of cytosolic and mitochondrial ROS (Ali et al. 2021).

Altogether, bidirectional communication between AT and cells of the cardiovascular system controls vascular homeostasis but also, when imbalanced, induce pathophysiological alterations.

A summary of the main features in the interplay between AT with liver, skeletal, muscle, pancreas, and the cardiovascular system can be found in Fig. 2.

3.5 Interaction Between Gut Microbiota, Liver, and AT

Under physiological conditions, the intestinal barrier is almost impermeable, but, during obesity, poor dietary habits favor a modification of the bacterial populations of the intestines. Indeed, a depletion of the bacterial richness as well as an increase of certain phyla such as Firmicutes and a decrease of others such as Bacteroidetes is observed in obese patients (Le Chatelier et al. 2013) as well as in human NAFLD and non-alcoholic steatohepatitis (NASH) (Loomba et al. 2017). These modifications favor increased lipid storage in liver, AT expansion, and TNFα or interferon gamma (IFNγ) production evoking an alteration of the epithelium and the passage of

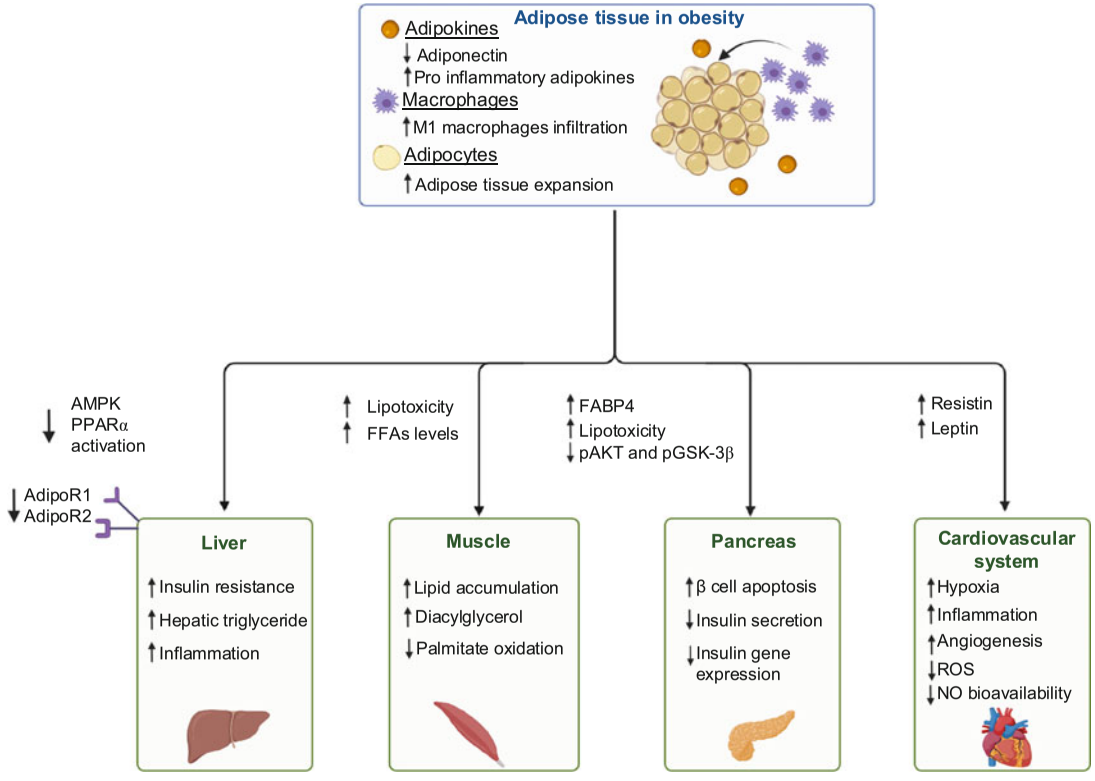

Fig. 2 Crosstalk between adipose tissue and several organs in obesity. Description of pathophysiological modifications in adipose tissue and their repercussion on liver, skeletal muscle, pancreas, and the cardiovascular system. *AMPK* AMP-activated protein kinase, *FABP4* Fatty acid-binding protein 4, *FFAs* Free fatty acids, *GSK-3β* Glycogen synthase kinase 3 beta, *NO* Nitric oxide, *PPARα* Peroxisome proliferator-activated receptor alpha, *ROS* Reactive oxygen species

microbial antigens or molecules such as LPS and SFAs (Madara and Stafford 1989; Clemente-Postigo et al. 2012) into the circulation. The presence of metabolic endotoxins in the bloodstream results in low-grade systemic inflammation which can alter the function of many organs through the activation of TLR4 pathway and ROS production (Cani et al. 2007). This mechanism maintains a pro-inflammatory loop favoring the development of metabolic syndrome and NAFLD (Baker et al. 2010; Fig. 3). LPS can be carried by bacterial EVs that alter the intestinal barrier by targeting tight junction proteins and increasing intestinal permeability leading to liver injuries via the activation of the LPS/TLR4 pathway (for review, see Villard et al. 2021) and impair glucose metabolism and insulin signaling in AT and skeletal muscle (Choi et al. 2015).

In isolated human abdominal subcutaneous adipocytes, LPS treatment promoted low-grade inflammation not only by activating TLR receptors but also by promoting the production of cytokines like TNFα and IL-6 (Creely et al. 2007). In a mouse model, the presence of a gut microbiota increased macrophage accumulation and LPS-dependent polarization toward the pro-inflammatory M1 phenotype in white AT (Caesar et al. 2012). Moreover, in humans, dietary treatments increasing SCFAs from microbiota reduced the production of pro-inflammatory adipokines such as IL-6 and IL-1β (Roager et al. 2019). Treatment of mice with tributyrin to mimic SCFA action reduced AT inflammation which was associated with an increase of M2 macrophages and regulatory T cells (Sato et al. 2020).

Altogether, these data suggest that metabolic endotoxemia, in particular, and pro-inflammatory gut microbiota in general influence AT function in obesity, mainly, by increasing inflammation (Clemente-Postigo et al. 2019). However, some studies show that the development of obesity and its associated AT inflammation, impaired glucose tolerance and liver steatosis are independent of gut microbiota, since germ-free and conventional mice are similar prone to these effects when fed with a high-fat diet (Moretti et al. 2021; Eckel 2021).

4 Perspective: Taming Inter-Organ Mediators to Control Obesity?

Lifestyle interventions together with bariatric surgery remain in most of the cases the best solution for obese patients. In the last 30 years, intensive research on AT contribution to metabolic diseases has led to exciting discoveries. However, identifying new targets to develop pharmacological strategies to combat obesity has proven hitherto unsuccessful. Therefore, a deep understanding of inter-organ crosstalk in the pathogenesis of obesity and T2D as well as the changes of organ communication among diet or exercise can help us identify novel therapeutic targets to treat and prevent metabolic diseases and its complications.

The exciting results of the adiponectin receptor agonist AdipoRon at preclinical level have not been translated into clinical studies. At the moment, FGF21 remains one of the few organokines that is under clinical investigation for the treatment of obesity, T2D, and complications like NAFLD. Several analogues and FGF21

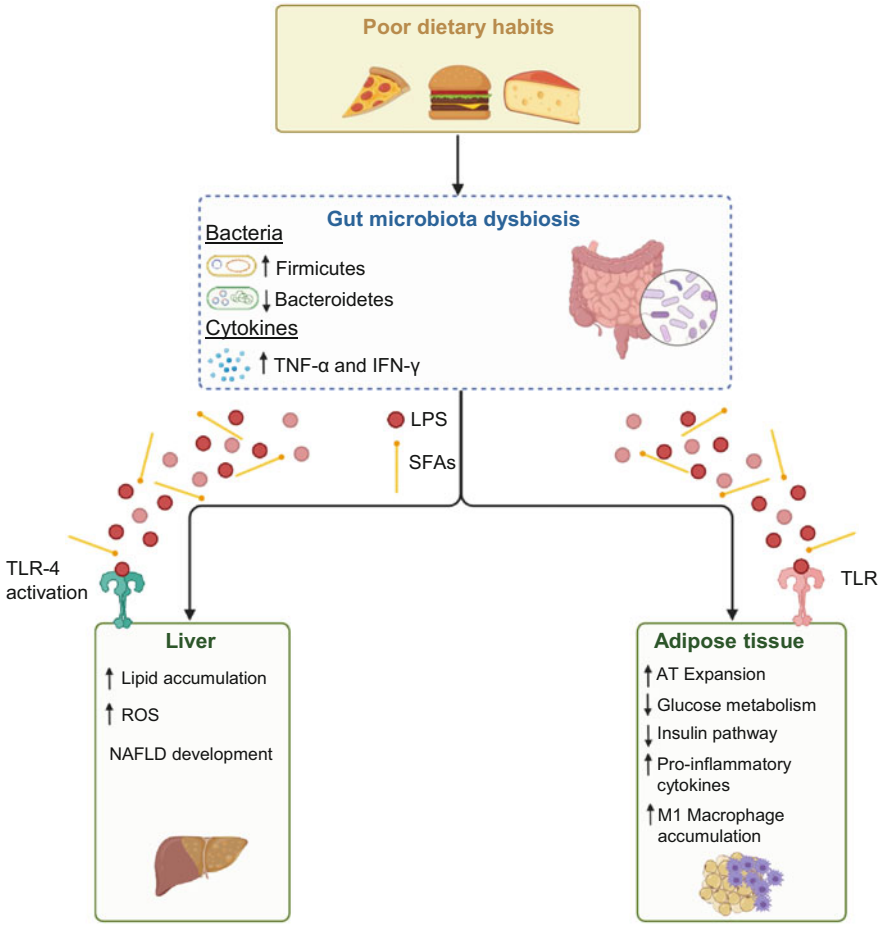

Fig. 3 The impact of gut microbiota dysbiosis on liver and adipose tissue. Poor dietary habits favor an increase of bacteria from certain phyla such as Firmicutes and a decrease of others such as Bacteroidetes. This imbalance favors TNF-α or IFN-γ production resulting in an alteration of the intestinal epithelium and the passage of microbial antigens or molecules such as LPS and SFAs in the circulation. The presence of metabolic endotoxins in the bloodstream results in a low-grade systemic inflammation which can alter the function of the liver through the activation of TLR pathways in the liver and the AT. This mechanism maintains the pro-inflammatory loop favoring the development of NAFLD as well as AT dysfunction. TNF-α and Interferon-γ production. *AT* adipose tissue, *IFN-γ* Interferon-γ, *LPS* Lipopolysaccharide, *NAFLD* Non-alcoholic fatty liver disease, *ROS* Reactive oxygen species, *SFA* saturated fatty acids, *TLR-4* Toll-like receptor-4, *TNF-α* Tumor necrosis factor-α

receptor agonists have undergone clinical trials bringing mixed results (as reviewed in Geng et al. 2020).

Although the impact of bariatric surgery on gut microbiota has been demonstrated, the validity of microbiota-based therapies to combat obesity and insulin resistance remains unclear (Wang et al. 2021b, Hanssen et al. 2021).

Several preclinical studies showed promising results for adipose-derived EVs from lean mice reducing AT inflammation and improving insulin sensitivity in the recipient obese mice (Huang and Xu 2021). The potential clinical use of EVs still seems technically challenging in terms of isolation, drug loading, large-scaling, and clinical grade production. Artificial engineered EVs with selected bioactive cargos might represent future alternatives to natural EVs for targeted drug delivery. Although the advances in –omics has led to in-depth characterization of the secretome of organokines, their precise interplay, biological function, and therapeutic potential require further research (Liu et al. 2019).

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Islet Inflammation and β Cell Dysfunction in Type 2 Diabetes

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Contents

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Abstract

Pancreatic islets are the body's central rheostat that regulates glucose homeostasis through the production of different hormones, including β cell-derived insulin. During obesity-induced type 2 diabetes (T2D), islet β cells become dysfunctional and inadequate insulin secretion no longer ensures glycemic control. T2D is associated with a chronic low-grade inflammation that manifests in several metabolic organs including the pancreatic islets. Growing evidence suggests that components of the innate immune system, and especially macrophages, play a crucial role in regulating islet homeostasis. Yet, the phenotypes and functions of islet macrophages in physiology and during T2D have only started to attract attention and remain unclear. In this review, the current knowledge about islet inflammation and macrophages will be summarized in humans and rodent models. Recent findings on the cellular and molecular mechanisms involved in islet remodeling and β cell function during obesity and T2D will be discussed.

Keywords

Inflammation · Insulin · Macrophages · Pancreatic islets · Type 2 diabetes · β Cells

1 Introduction

The endocrine system is the body's central rheostat that allows the maintenance of glycemic control through the production of different hormones. The pancreatic islets are micro-organs that constitute the endocrine pancreas and are distributed throughout the exocrine stroma. In the fed state, β cells, representing around 70% of the islet cells in mice and 50% in humans, secrete the amount of insulin required for optimal glucose uptake by the body's cells, lowering the glycemia. Normoglycemia is thus achieved by the balanced interplay between the amount of secreted insulin and the degree of insulin action. Failure to keep this balance under control underlies type 2 diabetes (T2D). T2D is a multifactorial and progressive disease associated with an impaired insulin action (a condition termed insulin resistance) coupled with a defect in β cell insulin secretion. T2D, which accounts for around 90% of all diabetes cases (Saeedi et al. 2020), predisposes to long-term life-threatening consequences including vascular damages, kidney and liver diseases, cancers, and cardiovascular complications. As such, T2D represents a major global illness burden in our ageing societies, affecting more than 450 million people worldwide (Saeedi et al. 2020).

For two decades now, obesity, insulin resistance and T2D have been linked to a state of low-grade, non-infective (i.e., "sterile") systemic chronic inflammation. Inflammation is now recognized as a major etiological component of a variety of health problems, including metabolic decline (Donath and Shoelson 2011; Furman et al. 2019). Numerous studies have described elevated circulating levels of acute phase proteins and typical pro-inflammatory molecules in T2D patients, suggesting

complex immune activation (Donath and Shoelson 2011). Inflammatory processes are also occurring locally in insulin-sensitive tissues including the liver and the white adipose tissue. Specific upregulation of genes encoding inflammatory factors such as Tumor Necrosis Factor (*Tnf*) have become key features of enlarged adipose tissue (Gregor and Hotamisligil 2011). Adipose tissue inflammation is associated with a marked accumulation of immune cells, especially the innate immune cell macrophages, within the visceral adipose tissue during obesity and may contribute to adipocyte stress and insulin resistance (Donath and Shoelson 2011). More recently, macrophages were also shown to reside inside pancreatic islets and seminal work has reported the accumulation of macrophages in obese and T2D islets (Ehses et al. 2007; Homo-Delarche et al. 2006). Yet the nature of islet inflammation and its impact on β cell homeostasis remain unclear.

The present review will thus explore the role of islet inflammation and mainly macrophages in human and rodent models of insulin resistance and T2D. Special attention will be given to selected most recent work and discussion will address pending questions and future perspectives in the field of islet immunometabolism.

2 T2D Etiology and Pathogenesis of β Cell Failure

2.1 Definition of T2D

In comparison with Type 1 Diabetes (T1D) that is characterized by an autoimmune destruction of β cells, T2D is a progressive disease that combines loss of adequate β cell insulin secretion on a background of insulin resistance. Indeed, T2D typically progresses from a state of prediabetes (that is, insulin resistance compensated by β cell hyperinsulinemia sufficient to maintain euglycemia) to a state of diabetes (which results from exhausted β cells that can no longer compensate for the insulin resistance) and consequently, the establishment of marked hyperglycemia (Kahn 1998). T2D is therefore a disease of inadequate insulin secretion in response to a degree of insulin sensitivity. T2D is currently diagnosed based on plasma glucose criteria: either the fasting plasma glucose value (>126 mg/dL) or the 2-h plasma glucose value during a 75 g oral glucose tolerance test (>200 mg/dL), or with the glycated hemoglobin (HbA1c) value (>6.5%) reflecting long-term blood glucose, according to the American Diabetes Association.

The current hypothesis is that T2D is the result of multifactorial etiologies where a mixture of genetic and environmental factors such as obesity, Western diet, a sedentary lifestyle, and aging are the underlying mechanisms (Sirdah and Reading 2020). Genome-wide association studies are providing an ever-increasing number of susceptibility variants in T2D and related complications. Yet, these variants only explain a small proportion of the total T2D heritability (Prasad and Groop 2015). Importantly, and despite the fact that insulin resistance is the main etiological factor in T2D development, the variants are mainly related to β cell function and insulin secretion rather than insulin action per se (Prasad and Groop 2015; Sirdah and Reading 2020). Thus, β cell failure remains at the center of the pathogenesis and

mainly determines the onset of T2D. It remains unclear and still debated whether this β cell failure is the result of compromised β cell function (i.e., glucose-induced insulin secretion by each individual β cell), reduced β cell mass (i.e., the total number of β cells within a pancreas), and/or the combination of both (Meier and Bonadonna 2013). Of note, β cell mass is tightly determined by the balance between β cell death (apoptosis/necrosis) and birth (through possible cell replication, neogenesis, and/or transdifferentiation).

2.2 β Cells at the Center of T2D

β cell compensatory response to insulin resistance has been well documented in rodents and involved a massive increase in β cell mass due to cell proliferation and hypertrophy (Aguayo-Mazzucato and Bonner-Weir 2018). In humans as well, several reports suggest a correlation between β cell mass and the body mass index or insulin resistance (Butler et al. 2003; Mezza et al. 2019). Thus, human β cells are also able to compensate for insulin demands, although the effect remains modest when compared with rodents and the mechanisms of β cell plasticity are still debated (Mezza et al. 2019). In T2D, several lines of evidence suggest a reduction in β cell number in the range of 10–60% in lean and obese patients compared with non-diabetic individuals, probably due to increased β cell apoptosis (Butler et al. 2003; Meier and Bonadonna 2013; Sasaki et al. 2021). In parallel, T2D patients and ex vivo T2D islets display impaired β cell function as defined by increased basal insulin and proinsulin release and loss of the first-phase insulin secretion in response to glucose (Butcher et al. 2014; Cohrs et al. 2020; Marselli et al. 2020; Solimena et al. 2018). Importantly, this β cell dysfunction is already observed in prediabetic individuals (Cohrs et al. 2020; Weaver et al. 2021). A growing body of evidence also suggests that T2D is associated with a process of β cell dedifferentiation that may contribute to β cell alteration (Hunter and Stein 2017). Thus, despite important interindividual variability, these studies altogether suggest that there may be progressive decline of β cell mass with duration of diabetes concurrent with early β cell dysfunction that contributes to the deterioration of glucose homeostasis. With respect to animal T2D models, reduced β cell mass and insulin secretion defect are also observed in the spontaneous T2D Goto-Kakizaki (GK) rats and leptin receptor deficient *Db*/*Db* mice but not in the insulin resistant diet-induced obese C57BL/6 and leptin deficient *Ob*/*Ob* mice (Kleinert et al. 2018).

The events that initiate β cell demise in T2D may involve different, but synergetic, mechanisms including lipotoxicity and glucotoxicity, endoplasmic reticulum and oxidative stress, and local hypoxia that are discussed elsewhere (Gregor and Hotamisligil 2011). An additional component that may be triggered by these mechanisms and in turn contributes to β cell damage is inflammation. T2D is associated with a chronic low-grade inflammation and it is gaining more and more attention that islets represent a target tissue. Based on an immune insulitis criterion, analyses could not discriminate between the pancreata retrieved from patients with T1D from those with T2D, confirming that islet inflammation is now part of the

etiology of T2D (Lundberg et al. 2017). Recently, a novel stratification identified five subgroups of diabetic patients differing in disease progression and risk of diabetic complications (Ahlqvist et al. 2020). Interestingly, levels of circulating inflammatory biomarkers were associated with T2D patients characterized by pronounced insulin resistance (Herder et al. 2021). These observations suggest that in this specific diabetic subgroup, inflammatory processes may particularly contribute to the disease progression and β cell dysfunction. Further studies should investigate the status of islet inflammation in this new clustering approach.

3 Islet-Resident Macrophages at Homeostasis

A pioneering study in the early 90s described innate immune macrophages inhabiting all endocrine organs in mice under steady-state (Hume et al. 1984). Macrophages populate healthy mouse islets as early as the perinatal stages and represent more than 80% of total intra-islet immune cells, with a number of around 2–10 macrophages per islet (Banaei-Bouchareb et al. 2004; Calderon et al. 2015; Dalmas et al. 2017). Although scarce relative to macrophages, other immune cell types were reported to also reside inside mouse and human islets including T and B cells and few innate lymphoid cells (ILC) such as NK cells and ILC2 (Dalmas et al. 2017; Denroche et al. 2021; Radenkovic et al. 2017). Thus, islet immunity is mainly driven by resident macrophages. These macrophages display two specific localizations: around the islets (named peri-islet macrophages) and inside the islets (named intra-islet macrophages). In adult mice, islet-resident macrophages originate from definitive hematopoiesis and strongly depend on Colony Stimulatory Factor 1 (CSF1, also known as Macrophage-CSF) for their survival. They are long-lived and self-maintained by in situ proliferation with minimal contribution from circulating monocytes (Banaei-Bouchareb et al. 2004; Calderon et al. 2015; Carrero et al. 2017; Ying et al. 2019). In humans, resident immune cells are also observed within islets of healthy donors, with the presence of macrophages and few T, B, and mast cells but at lower frequencies when compared with rodent islets (Butcher et al. 2014; Ehses et al. 2007; Martino et al. 2015; Nordmann et al. 2017; Richardson et al. 2009).

Research groups have only recently started to explore the phenotype of islet macrophages, mainly in mice. Islet macrophages express typical myeloid markers such as Cluster of Differentiation (CD)64 and F4/80. In metabolism, macrophage phenotype was initially described based on the M1/M2 paradigm of macrophage activation, extrapolated from in vitro polarization studies (Martinez and Gordon 2014). In white adipose tissue, it was proposed that macrophages switch from a quiescent (M2-like) phenotype to a more pro-inflammatory (M1-like) activation during obesity (Gregor and Hotamisligil 2011). Recent studies have now revisited these early interpretations showing that adipose tissue macrophages rather acquire a lipid-handling "metabolically-activated" phenotype during weight gain (Marcelin et al. 2021). In mouse islets, macrophages disrupt even more this original "M2" to "M1" shift associated with metabolic decline. Indeed, they appear to already adopt

an activated phenotype at steady-state. Islet macrophages constitutively harbor M1-like macrophage markers including CD11c and the Major Histocompatibility Complex (MHC) class II on their membrane, but do not express M2-like markers such as the Mannose Receptor CD206. Islet macrophages also show high expression levels of the typical pro-inflammatory mediators Interleukin(*Il)1b* and *Tnf*, suggesting a surprising pro-inflammatory profile under homeostasis (Calderon et al. 2015; Ferris et al. 2017; Weitz et al. 2020). In humans, islet macrophages express the myeloid markers CD14 and ionized calcium-binding adaptor molecule 1 (IBA1) as well as CD206, which distinguish them from mouse islet counterparts (Weitz et al. 2020). The phenotype and activation status of human islet macrophages remain so far largely unexplored. Using macrophage-depleting agents in lean and obese/T2D islets, macrophages were shown to be the main source of the pro-inflammatory factor *Il1b* and *Tnf* in mouse islets (Calderon et al. 2015; Chan et al. 2019; Nackiewicz et al. 2014) and only *IL1B* in humans islets (He et al. 2019).

Stromal macrophages residing in the exocrine part of the pancreas (surrounding islets) show a contrary M2-like quiescent phenotype (Calderon et al. 2015). Notably, mouse macrophages located in the islet periphery stained for CD206 in a pattern that clearly delineated the islet capsule and showing that exocrine macrophages are different from islet macrophages (Weitz et al. 2020). These data support the hypothesis that the islet environment may specifically shape this atypical macrophage activation state. Indeed, tissue-resident macrophages are known to possess a conserved and specific transcriptomic signature that is influenced by their ontogeny and the local microenvironment, also known as niche (Guilliams et al. 2020). The islet niche may thus be characterized by a physiological activation and maintenance of their resident macrophages. Further studies are warranted to fully explore the islet phenotype in mice and human, especially in light of new single-cell genomic approaches.

4 Islet Inflammation and Immune Cells in T2D Patients

4.1 Immune Cells in T2D Islets

Low-grade inflammation has been observed in patients with T2D at the systemic level and in metabolic tissues (Donath et al. 2019). In the early 2000s, hypotheses that inflammatory processes, such as those involved in autoimmune diabetes, may also play a role in β cell dysfunction in T2D began to develop. In 2007, Ehses and colleagues observed for the first time that $CD68^+$ macrophages reside in human pancreatic islets and that T2D patients display increased number of macrophages in islets compared to non-diabetic subjects. Macrophages were frequently located among the endocrine cells within T2D islets, whereas they were generally distributed more in the periphery of control islets. Their localization did not correspond to apoptotic β cells in T2D pancreata (Ehses et al. 2007). Increased number of total $CD45^+$ immune cells and macrophages in T2D was further confirmed by several publications as summarized in Table 1. Notably, in a cohort of T2D patients, 28%

Table 1 Immune cells in islets of T2D patients compared non-diabetic subjects

Reference	Approach to sample collection	Number of samples	Method	Observation in T2D compared to ND
(Ehses et al. 2007)	Organ donors, necropsy, and surgery patients	7 ND 9 T2D	IHC	↑ Number of $CD68^+$ macrophages per islet → Granulocyte and $CD3^+$ T cell number
(Richardson et al. 2009)	Organ donors and autopsy patients	16 ND 15 T2D	IHC	↑ Number of $CD68^+$ macrophages per islet
(Butcher et al. 2014)	Organ donors	15 ND 5 T2D with high insulin-secreting islets 5 T2D with low insulin-secreting islets	FACS	↑ Number of $CD45^+$ immune cells per islet in high insulin-secreting islets → Number of $CD45^+$ immune cells per islet in low insulin-secreting islets ↑ Proportion of $CD20^+$ B cells (% of $CD45^+$ cells) → Proportion of $CD3^+$ T cells (% of $CD45^+$ cells) → Proportion of $CD11c^+$ myeloid cells (% of $CD45^+$ cells)
(Rodriguez-Calvo et al. 2014)	Organ donors	15 ND 11 T2D	IHC	↑ Number of $CD8^+$ cytotoxic T cells only in the periphery of islets only
(Martino et al. 2015)	Organ donors	7 ND 7 T2D	Electron microscopy	↑ Number of macrophages per islet ↑ Number of lymphocytes per islet → Number of mast cells per islet
(Nordmann et al. 2017)	Organ donors	16 ND 17 T2D	IHC	↑ Number of $CD45^+$ macrophages per islet and per islet area
(Lundberg et al. 2017)	Organ donors	44 ND 50 T2D	IHC	28% of T2D patients had insulitis (2013 criterion: ≥3 islets with ≥15 $CD45^+$ cells) 0% of ND had insulitis
(Horii et al. 2020)	Pancreatectomy patients (with a pre-surgery oGTT)	21 NGT 15 IGT 24 T2D	IHC	↑ Number of $CD68^+$ macrophages per islet
(Wu et al. 2021)	Organ donors	13 ND 16 T2D	IMC	↑ Number of intra-islet $CD68^+$ macrophages per islet area (but not peri-islet $CD68^+$ macrophages) ↑ Number of intra-islet and peri-islet $CD8^+$ T cells per islet area

(continued)

Table 1 (continued)

Reference	Approach to sample collection	Number of samples	Method	Observation in T2D compared to ND
				Both $CD68^+$ macrophages and $CD8^+$ T cells were HLD-DR^{high}

ND non-diabetic, *T2D* Type 2 diabetic, *NGT* Normal glucose tolerant, *IGT* Impaired glucose tolerant, *HFD* High fat diet, *IHC* immunohistochemistry, *FACS* flow cytometry, *IMC* Imaging Mass Cytometry

were diagnosed with insulitis using the 2013 (T1D-adapted) definition of islet immune insulitis (defined as ≥3 islets with ≥15 $CD45^+$ cells per pancreas section). Using this criterion, analyses could not discriminate between pancreata retrieved from patients with T1D (31% of them had insulitis) from those with T2D (Lundberg et al. 2017). The intensity of inflammation appeared heterogeneous in a single T2D pancreas between the different lobes and a third of T2D patients also displayed fibrotic areas enriched in immune cells (Lundberg et al. 2017). Macrophages are the main immune cells observed in human islets but other cell types were also detected. With regard to their association with T2D, discrepancies exist. Publications from Ehses et al. and Lundberg et al. did not observe any infiltration of $CD3^+$ T cells in islets, whereas the number of total lymphocytes (as assessed by electron microscopy), intra-islet $CD20^+$ B cells and peri-islet $CD8^+$ cytotoxic T cells were reported to be increased in T2D islets compared to non-diabetic controls (Butcher et al. 2014; Martino et al. 2015; Rodriguez-Calvo et al. 2014). More recently, using advanced multiplexed imaging technique, Wu and colleagues observed increased intra-islet macrophages and $CD8^+$ T cells in T2D pancreata compared to non-diabetic subjects but not other types of immune cells nor in exocrine tissue (Wu et al. 2021). Both macrophages and $CD8^+$ cells expressed high levels of HLA-DR (the human MHCII surface receptor) and were more likely to be in contact with β cells in T2D. Thus, while it is clear that macrophages accumulate inside islets of T2D patients, further studies should clarify whether other components of the immune system are also at stake. In particular, islet $CD8^+$ T cells require further attention as their number is affected during disease progression and that Radenkovic et al. identified a specific resident memory $CD8^+$ population in healthy human islets (Radenkovic et al. 2017; Rodriguez-Calvo et al. 2014; Wu et al. 2021).

4.2 Transcriptomic Analyses of T2D Islets

Transcriptomic analyses of human islets or laser-capture microdissected β cells show that T2D is associated with increased gene expression of chemokines such as *IL8*, *CCL2,* and *CCL13* and cytokines such as *IL1B*, *TNF, and IL24* (Blencowe et al. 2021; Boni-Schnetzler et al. 2008; Butcher et al. 2014; Igoillo-Esteve et al. 2010; Marselli et al. 2020). Macrophage markers such as *APOE* and CD163L1 were also

found overexpressed in T2D islets, supporting their accumulation during T2D (Marselli et al. 2020). Increased gene expression of *CCL2* and *TNF* in T2D Islets was inversely correlated to ex vivo insulin secretory function, suggesting a link between inflammation and β cell dysfunction (Butcher et al. 2014). By analyzing global gene expression in human pancreatic islets, a gene module enriched for interleukin-1-related genes was associated with T2D traits and reduced islet insulin secretion. For instance, they found that the gene expression of the IL-18 receptor *IL18R1* in islets correlated the most with glucose-induced insulin secretion and HbA1c of the islet donors (Mahdi et al. 2012). Another study shows that islet gene expression of the decoy IL-1 receptor type II *IL1R2* was differentially expressed between T2D and non-diabetic individuals and was positively correlated to Hb1Ac while negatively correlated to insulin secretion (Taneera et al. 2012). These transcriptomic human studies altogether demonstrate that an immune response is occurring in islets of T2D patients, that is characterized by innate immune cell activation, interleukin production and chemotaxis. Confirming histology observations, islet inflammation seems to be mainly driven by myeloid cells and orchestrated by components of the IL-1 family in diagnosed T2D. It remains to be seen whether islet inflammation develops before the diagnosis of T2D, during the under-explored prediabetes period.

4.3 Islet Inflammation During the Progression of T2D

A recent paper by Wigger and colleagues explored the gene expression of β cell-enriched islets isolated by laser capture from metabolically phenotyped patients undergoing pancreatectomy, including controls, prediabetic and T2D patients. Notably, they show that islets from patients with prediabetes and diabetes displayed a progressive upregulation of genes functionally related to cell signaling (including the NFκB-, Toll Like Receptors-, Transforming Growth Factor β- and TNF-related pathways) and immune responses (including pathways of antigen processing and presentation, T cell infiltration and complement activation) compared to β cells isolated from non-diabetic subjects (Wigger et al. 2021). In the same way, islets isolated from prediabetic donors (characterized by a Hb1Ac between 5.7 and 6.4%) display an impaired insulin secretion and increased *IL1B*, *TNF*, and *IL6* (but not Interferon *IFNG*) gene and protein expression compared to normoglycemic controls (Weaver et al. 2021). Studying well-phenotyped patients, a progressive increase in $CD68^+$ macrophages per islet was noted in impaired glucose tolerant, and more so in T2D patients compared to normal glucose tolerant subjects; yet, significance was only achieved between controls and T2D islets (Horii et al. 2020). Thus, islet inflammation as defined by macrophage activation and accumulation may contribute to the progressive decline of β cell function, preceding the onset of T2D (Wigger et al. 2021). The fact that an inflammatory signature may already be detectable at the prediabetes stage is to be noted, considering the development of anti-inflammatory therapies (Donath et al. 2019). However, other studies did not detect any obvious signature of local inflammation in islets or in β cell transcriptomes in

microarray-based analysis (Solimena et al. 2018) and especially in pioneering single-cell RNA sequencing (scRNAseq) analyses (Lawlor et al. 2017; Segerstolpe et al. 2016; Wang et al. 2016; Xin et al. 2016).

4.4 Islets in the Single-Cell Era

Since 2016, novel single-cell approaches allowed the generation of comprehensive maps of islet cellular heterogeneity and individual cellular changes during aging and T2D (Avrahami et al. 2020; Lawlor et al. 2017; Segerstolpe et al. 2016; Wang et al. 2016; Xin et al. 2016). However, these first studies did not allow the isolation and study of high number of cells (from 619 to 1,492 cells isolated from at least eight pooled individual islet preparations). Accordingly, scRNAseq studies did not detect any islet-resident immune cells due to their low frequencies, with the exception of Segerstolpe et al. who annotated seven mast cells and five macrophages in their analysis. T2D islet scRNAseq datasets show a reduced number of β cells analyzed, which may reflect their increased fragility and susceptibility to loss during cell sorting (Lawlor et al. 2017; Segerstolpe et al. 2016). Therefore, early scRNAseq studies are of special interest but may have not covered the spectrum of β cell heterogeneity and immune cell activation. Indeed, in a healthy islet at a given time, β cells were shown to have different functional states and in the autoimmune nonobese diabetic (NOD) mice, some β cells are known to resist immune attack (Dominguez-Gutierrez et al. 2019; Rui et al. 2017). These observations suggest that β cells are a heterogenous population that may differently respond to inflammation with possible subsets of intact, passive, and inflamed β cells. Advances in scRNAseq technologies may soon improve these technical limitations and future studies are expected to provide insight into the spectrum of islet cell response during T2D.

Interestingly, by comparing scRNAseq data of islets isolated from individuals of different ages (one newborn, five toddlers, two adolescents, and four adults), Avrahami and colleagues showed that β cell undergo a natural maturation process during aging, identifying a set of juvenile genes that are repressed during adulthood. Importantly, in T2D patients, most of β cells seem to return to this immature expression profile by de-repressing juvenile genes while activating gene sets typical of the exocrine compartment (Avrahami et al. 2020). This is in concordance with the observation that T2D is characterized by a relaxation of β cell gene identity through reduced gene silencing by the Polycomb-Repressive Complex 2 (Lu et al. 2018). Importantly, biological pathways associated with TNF signaling and inflammatory response were decreased with age and upregulated in T2D patients (Avrahami et al. 2020). Thus, islet inflammation may be a dynamic process in close correlation with the degree of β cell maturation. During the progression of T2D, inflammation may impair specific epigenetic mechanisms necessary for the maintenance of terminal β cell differentiation. Moreover, an inflammatory signature is also observed in acinar cells of T2D patients, suggesting that the exocrine tissue may also play a role in the inflammatory mechanisms involved in T2D pathogenesis (Segerstolpe et al. 2016).

5 Islet Homeostasis During Metabolic Stress in Rodents

5.1 Islet Inflammation and Immune Cells in Obese and Diabetic Rodents

Because fibrotic lesions are observed in the diabetic GK rats and that immunity is usually associated with fibrosis, it was predicted that an inflammatory reaction may be occurring inside islets. Indeed, GK islets show increased expression of genes involved in extracellular matrix, inflammation, and immune response including the macrophage markers *Lgals3* and *Cd74* compared to the Wistar rat control islets (Ehses et al. 2007; Homo-Delarche et al. 2006). Increased inflammation, specifically innate immune factors such as *Tnf*, *Il1b,* and *Cxcl1*, was confirmed in islets isolated from various obese and/or diabetic rodent models (Chan et al. 2013; Eguchi et al. 2012; Ehses et al. 2007; Hasnain et al. 2014; Jourdan et al. 2013). One publication did not observe increased *Il1b* gene expression or the IL-1β-processing inflammasome NLRP3 activation in *Db*/*Db* islets (Kammoun et al. 2018).

Further immunohistochemical analyses reveal the increased abundance of $MHCII^+$ and $CD68^+$ macrophages in and around the islets of diabetic GK rats compared to lean counterparts (Ehses et al. 2007; Homo-Delarche et al. 2006). This seminal discovery of macrophages in rat islets was also later confirmed by other groups in complementary models of obese and/or T2D mice and rats using immunohistochemistry and flow cytometry techniques as recapitulated in Table 2. Of note, obesity did not promote T and B cells accumulation in islets isolated from high fat diet (HFD)-fed mice (Denroche et al. 2021).

In order to explore the impact of progressive glucotoxicity on β cell homeostasis, the model of rat partial pancreatectomy, that is characterized by a defect in glucose-induced insulin secretion, was used to trigger a progressive mild hyperglycemia. This experimental setup allowed the analysis of islet gene expression isolated from animals displaying a spectrum of glycemia. Surprisingly, pathways associated with inflammation were significantly enriched in hyperglycemic islets with increased gene expression of innate immune cytokines and chemokines including *Il1b*, TLRs and NFκB activation, antigen presentation and potential macrophage markers including *Cd68*, *Cx3cr1,* and *Cd74* (Ebrahimi et al. 2020). Interestingly, this islet immune response develops soon after the pancreatectomy surgery and intensifies during the course of hyperglycemia. Further work is warranted to fully explore the dynamic presence and role of macrophages in this model.

5.2 Phenotype of Mouse Islet Macrophages During Obesity and T2D

Two studies suggest the existence of different subsets of islet macrophages, discriminating the resident from the pro-inflammatory or pathological ones. The islet-resident $CD11b^+Ly6C^-$ or $F4/80^{hi}CD11c^{lo}$ macrophages predominate at steady-state and the $CD11b^+Ly6C^+$ or $F4/80^{low}CD11c^{high}$ macrophages accumulate

Table 2 Immune cells in islets of obesity and/or T2D rodent models compared to controls

Reference	Rodent model	Method	Observation in obese/diabetic animal compared to controls
(Ehses et al. 2007)	Obese HFD-fed C57BL/6J mice CD-fed controls (8 weeks)	IHC	↑ Number of $CD11b^+$ macrophages per large islet
	Diabetic nonobese GK rats Wistar controls (2 months old)	IHC	↑ Number of $CD68^+$ macrophages per islet
	Obese diabetic *Db*/*Db* mice *Db*/+ (9 weeks old)	IHC	↑ Number of $CD11b^+$ macrophages per islet
(Agudo et al. 2012)	Obese HFD-fed C57BL/6SJL mice CD-fed controls (16 weeks)	IHC	↑ Number of $Mac2^+$ macrophages per islet
(Eguchi et al. 2012)	Obese diabetic *Db*/*Db* mice *Db*/+ (8 weeks old)	FACS	↑ Frequencies of $CD11b^+Ly6c^+$ monocytes/macrophages among islet live cells
	HFD-fed KKay mice Kkta controls	FACS	↑ Frequencies of $CD11b^+Ly6c^+$ monocytes/macrophages among islet live cells
(Jourdan et al. 2013)	Leptin-resistant obese Zucker male rat versus lean controls (8 weeks old)	IHC	↑ Number of $CD68^+$ macrophages per islet (no count)
(Cucak et al. 2014)	Obese diabetic *Db*/*Db* mice *Db*/+ (8 weeks old)	FACS	↑ Number of $CD68^+F4/80^+$ and $CD68^+F4/80^-$ macrophages per islets
(Chan et al. 2019)	Obese diabetic *Db*/*Db* mice *Db*/+ (16 weeks old)	FACS	↑ Frequencies of $CD11b^+F4/80^+$ macrophages among islet live cells
(Ying et al. 2019)	Obese HFD-fed C57BL/6J mice CD-fed controls (1 to 30 weeks)	IHC	↑ Number of $CD45^+$ immune cells per islet ↑ Number of $CD11c^+$ macrophages per islet
(Chittezhath et al. 2019)	Obese diabetic *Db*/*Db* mice *Db*/+ (8, 12 and 16 weeks old)	IHC/ FACS	↑ Frequencies of $CD68^+$ and $F4/80^+$ macrophages among total islet cells ↑ Number of $CD11b^+F4/80^+MHCII^+$ macrophages per islet
(Denroche et al. 2021)	Obese HFD-fed C57BL/6J mice CD-fed controls (12 weeks)	FACS	→ Frequencies of total $CD45^+$ immune cells among islet live cells → Frequencies of $CD3^+$ T cells among islet live cells → Frequencies of $CD19^+$ B cells among islet live cells → Frequencies of $CD45^+CD3^-CD19^-$ cells (≈macrophages) among islet live cells

HFD High fat diet, *IHC* immunohistochemistry, *FACS* flow cytometry

inside islets during the course of HFD-induced obesity (Eguchi et al. 2012; Ying et al. 2019). While the $CD11b^{+}Ly6C^{+}$ macrophages are recruited from circulating monocytes, $F4/80^{low}CD11c^{high}$ macrophages were shown to proliferate in situ. This is in contradiction with other studies showing that all islet-resident macrophages are positive for CD11c even under steady-state (Calderon et al. 2015; Dalmas et al. 2017). Similarly, studies have shown that $CD11b^{+}F4/80^{+}$ macrophages accumulating inside diabetic *Db*/*Db* and HFD-fed obese islets were mainly $MHCII^{high}$ and $CD11c^{high}$ but negative for Ly6c, suggesting the absence of recruited monocyte-derived macrophages. Instead, islet macrophages were shown to proliferate much more under HFD and islet inflammation is mainly driven by in situ expanded macrophages (Chittezhath et al. 2019; Ying et al. 2019). Indeed, by transferring fluorescent monocytes in obese mice, Ying and colleagues observed that monocytes remained at the periphery of the islet capsule, without infiltration inside the islets and further differentiation into macrophages (Ying et al. 2019).

Discrepancies exist around islet inflammation in several mouse models of insulin resistance and T2D. Mouse islet macrophages already have a pro-inflammatory activated phenotype under steady-state, which raises the questions whether obesity can further alter their gene expression prolife as in adipose tissue (Gregor and Hotamisligil 2011). Gene expression analyses of $CD11b^{+}F4/80^{+}$ FACS-sorted macrophages isolated from *Db*/*Db*, *Ob*/*Ob* or HFD-fed C57BL/6 obese mice (5 and 16 weeks of HFD) did not show increased inflammatory response in those cells compared to lean counterparts. Instead, the basal islet macrophage activation profile was retained after metabolic stress with a tendency toward less *Il1b* and more of the IL-1 antagonist *Il1rn* gene expression, suggesting an overall decrease in IL-1β activity in obese islet macrophages (Calderon et al. 2015; Chan et al. 2019; Ying et al. 2019). In HFD-fed mice, the frequency of $CD45^{+}CD3^{-}CD19^{-}$ cells (which represent mainly macrophages) was not increased upon obesity (12 weeks HFD regimen) by flow cytometry (Denroche et al. 2021). Similarly, as compared to the diabetic *Db*/*Db* mice, inflammation and macrophage accumulation was not observed in insulin resistant but normoglycemic *Ob*/*Ob* obese mouse islets. This suggests that inflammation is not solely attributable to obesity and dyslipidemia (occurring in both *Db*/*Db* and *Ob*/*Ob* mice) but is specifically associated with β cell failure (Chan et al. 2013; Chan et al. 2019). Thus, the "M2" anti-inflammatory to "M1" pro-inflammatory shift that was initially accepted in adipose tissue macrophages during obesity may also not be applied to islet macrophages (Gregor and Hotamisligil 2011). In fact, these cells may actually play two roles at the same time: (1) they may support β cell response to metabolic stress during the compensation period (i.e., prediabetes stages) and (2) they may progressively contribute to their dysfunction during a decompensation period (i.e., diabetes stage).

6 Interactions Between Macrophages and β Cells in Islets

6.1 Cytokines and β Cell Function

T2D is associated with impaired β cell function as defined by increased fasting insulin secretion, decreased insulin secretion in response to glucose, and abnormal immature proinsulin release. To which extent islet inflammation contributes to β cell dysfunction has only just begun to be explored. Macrophages are the major source of cytokines in islets, especially IL-1β. Importantly, β cells have the highest expression of the signaling IL-1 receptor 1 (IL-1R1) of any other tissues, pointing to a specific IL-1β sensitivity in β cells (Boni-Schnetzler et al. 2009). While IL-1β may play a physiological role in promoting post-prandial insulin secretion (Burke et al. 2018; Dror et al. 2017), it has long been recognized that chronic exposure of islets to high levels of IL-1β is associated with β cell dysfunction and apoptosis in mice and humans (Maedler et al. 2002). Exposure of healthy islets to conditioned media of TLR2- and TLR4-activated macrophages greatly impairs their glucose-induced insulin secretion in a soluble mediator-dependent manner (Nackiewicz et al. 2014). Islets express all members of the IL-1 regulatory system, highlighting the need for subtle control of IL-1β signaling. As such, increased IL-1β signaling due to the β cell-specific deletion of the antagonist IL-1Ra disrupts β cell function (Boni-Schnetzler et al. 2018). The physiological insulinotropic effect of IL-1β is lost in islets isolated from T2D donors, showing that IL-1β may become only detrimental in the T2D context (Hajmrle et al. 2016). Accordingly, targeting innate inflammatory pathways improves glycemia and β cell function in rodents and results from anti-IL1β clinical trials have been very encouraging for the treatment of T2D (Donath et al. 2019).

Pro-inflammatory cytokines including IL-1β, IL-6, and TNF, but not IFNγ, are also able to repress β cell identity genes (Nordmann et al. 2017; Stancill et al. 2021). This β cell de-maturation or dedifferentiation is proposed to be one of the underlying causes of β cell failure in T2D and involves reduced expression of β cell identity genes and regression toward a progenitor-like state (Hunter and Stein 2017). IL-1-β-mediated gene repression in β cells was observed after only 6 h of treatment, suggesting that dedifferentiation is an immediate protective response to cytokine exposure rather than a long-term β cell decline mechanism (Stancill et al. 2021). Exposure of islet or the β cell line Min6 to macrophage conditioned media decreased *Pdx1* and *Ins1* gene expression in concomitant IL-1β-, Il-6- and TNF-dependent manners (Eguchi et al. 2012; Nackiewicz et al. 2014). Conversely, macrophage depletion in *Db*/*Db* islets restored key mature β cell gene expression, including Insulin2 (*Ins2*), Glucose transporter 2 (*Slc2a2*), Pancreatic and duodenal homeobox 1 (*Pdx1*), and *MafA* (Chan et al. 2019; Eguchi et al. 2012). Yet other work could not reproduce these observations (Chittezhath et al. 2019). As part of the de-maturation process, inflammatory cytokines also promote the occurrence of double insulin and glucagon-positive cells in islets, as observed in obese mice (Tesi et al. 2021; Ying et al. 2019). This switch from β to a β/α mixed phenotype may be a protective mechanism, to the detriment of insulin secretion, since α cells are incidentally found

to be less sensitive to stress-induced apoptosis (Marroqui et al. 2015). Depleting macrophages and neutralizing macrophage-derived pro-inflammatory mediators may be beneficial for preserving β cell identity and function in T2D islets.

6.2 Macrophages and β Cell Function

The impact of macrophages on insulin secretion was mainly addressed using macrophage-depleting agents such as liposomes-encapsulated clodronate that induces macrophage apoptosis once ingested, and the anti-CD115 neutralizing antibody targeting the CSF1 receptor. Depletion of macrophages improved the glucose-stimulated insulin secretion of islets isolated from HFD-fed obese mice, *Db*/*Db* and KKay diabetic mice (Chittezhath et al. 2019; Eguchi et al. 2012; Ying et al. 2019). Conversely, co-culture of Min6 cells with islet $CD11c^+$ macrophages isolated from obese mice impaired their function in an unclear cell–cell contact-dependent manner (Ying et al. 2019). In their article, Ying and colleagues propose that intra-islet macrophages phagocytose β cell insulin secretary granules, which may contribute to the impaired β cell insulin secretion in obese mice. In another study, islet macrophage depletion was not sufficient to restore insulin secretory function in *Db*/*Db* islets but significantly reduced the amount of proinsulin secretion, suggesting improved insulin processing (Chan et al. 2019). In humans, in contrast to the gene expression pattern of inflammatory factors, the number of $CD45^+$ immune cells was increased in T2D islets that have a preserved insulin secretion but not in markedly dysfunctional islets (Butcher et al. 2014). This suggests that early stages in T2D development may be accompanied by an influx of immune cells but not inflammation per se. Similarly, the presence of insulitis in T2D islets was not associated with further alteration of their insulin secretion (Lundberg et al. 2017). Thus, keeping in mind the dual role of IL-1β, immune cell infiltration in islets may be uncoupled with inflammation and that immune cells may primarily support β cell adaptation to metabolic stress, before switching to a deleterious inflammatory response eventually leading to T2D.

6.3 Macrophages and Islet Remodeling

In 2004, using the whole-body macrophage-deficient $Csf1^{op}/Csf1^{op}$ mice, a first study shows that macrophages are essential for β cell formation during embryonic development with reduced islet size (Banaei-Bouchareb et al. 2004). During weight gain and the development of insulin resistance in rodents, pancreatic islets increase their insulin production by increasing the β cell mass mainly through proliferation of pre-existing β cells (Aguayo-Mazzucato and Bonner-Weir 2018). Recent studies demonstrate that macrophage depletion in obese mouse models significantly decrease the size of islets and that macrophages isolated from obese islets (but not lean islets) promote β cell proliferation via the PDGFR signaling pathway (Chittezhath et al. 2019; Ying et al. 2019). In contrast, excess IL-1β signaling is

shown to inhibit β cell replication during obesity and aging (Boni-Schnetzler et al. 2018; Boni-Schnetzler et al. 2021). Similarly, combined genetic loss of Toll-like receptor (TLR)2 and TLR4 increases the replication of β cells, but not that of α cells, leading to enlarged β cell area and hyperinsulinemia in diet-induced obesity (Ji et al. 2019b). Since macrophages are the major contributor of IL-1β production in islets in response to TLR2 and TLR4 ligands (Nackiewicz et al. 2014), it suggests that elevated TLR signaling in macrophages during obesity may limit β cell proliferation through IL-1β production. Macrophage depletion also alters blood vessel density during obesity (Chittezhath et al. 2019). However, macrophage-depletion strategies did not discriminate between islet macrophages and macrophages present in other metabolic tissues. To specifically address the role of islet macrophages, islets depleted of macrophages or not were transplanted into HFD-fed mice. In the absence of macrophages, transplanted islets show a delay in revascularization, suggesting that islet macrophages contribute to the maintenance of a proangiogenic islet milieu for vascular integrity (Chittezhath et al. 2019). Thus, islet macrophages are necessary to ensure islet remodeling during metabolic stress. Yet, when TLR-activated, they may also contribute to β cell decline by limiting β cell mass and eventually compromising compensatory hyperinsulinemia during severe insulin resistance.

7 Other Parameters Regulating Islet Inflammation and β Cell Dysfunction

7.1 Islet Amyloid Deposition

Amyloid deposits are a histopathological feature of islets in 70–90% of T2D patients. The unique constituent of amyloid in islets is amylin or human islet amyloid polypeptide (hIAPP), a polypeptide hormone that is co-expressed with insulin. Once produced, mature hIAPP is co-packaged with insulin in secretory granules of β-cells and then co-released in response to glucose. In response to insulin resistance, the increased production of insulin is accompanied by augmented hIAPP levels. The progressive impairment of β cell processing machinery leads to the accumulation of unprocessed and misfolded hIAPP that form aggregates and contribute to β cell function in T2D (Kanatsuka et al. 2018). Species with amyloidogenic IAPP similar to humans, such as non-human primates and cats, share susceptibility to T2D, while those with non-amyloidogenic IAPP, such as rodents, do not. To explore the role of hIAPP in β cell dysfunction, a transgenic mouse expressing the hIAPP in β cells has been generated. When these mice are fed a HFD, amyloid deposition is observed in islets and is associated with impaired β cell function. In this transgenic mice, hIAPP deposition is associated with elevated gene expression of cytokines (*Il1b*, *Tnf,* and *Il6*) and chemokines (*Ccl2* and *Cxcl1*) and increased accumulation of macrophages around islet hIAPP aggregates (Masters et al. 2010; Meier et al. 2014; Westwell-Roper et al. 2011). Notably, during obesity, islet-resident macrophages, which are the main source of islet inflammation in response to hIAPP, possess more of a pro-inflammatory phenotype driven by the inflammasome NLRP3 (Masters et al.

2010; Westwell-Roper et al. 2011). Importantly, macrophage depletion in transgenic β cell hIAPP obese mice decreases islet inflammation and rescues glucose-induced insulin secretion (Westwell-Roper et al. 2011). In humans, islets from T2D patients that are positive for islet amyloid deposition show decreased β cell area and increased number of macrophages compared to T2D patients without overt sign of amyloid deposition (Kamata et al. 2014). Notably, the transcriptomes of islets transgenic for hIAPP (but not islets transgenic for rat IAPP) show striking similarities with the transcriptomes of human islets with prediabetes and T2D. Indeed, prediabetes/T2D human islets and β cell hIAPP mouse islets were both characterized by prominent inflammatory responses and dedifferentiation markers (Blencowe et al. 2021). Thus, inflammation is a hallmark of T2D islets and IAPP deposition may initiate this response through macrophage activation during the development of T2D.

7.2 Pancreatic Steatosis

Fat infiltration in the pancreas is a type of ectopic fat deposition similar to visceral fat accumulation and hepatic steatosis, typically occurring during weight gain. In the pancreas, lipids are mainly stored in Oil Red O- and perilipin-positive adipocytes which infiltrate the parenchyma, in and around islets, in mice and humans (Gerst et al. 2019). The link between pancreatic fat accumulation and β cell dysfunction remains unclear. Some studies demonstrate that pancreatic lipid content is higher in T2D patients compared to age- and BMI-matched non-diabetic subjects, while other studies did not find any correlations (Gerst et al. 2019). Interestingly, pancreatic fat content was independently associated with various aspects of β-cell function in non-diabetic men but not in T2D patients (Tushuizen et al. 2007). Similarly, pancreatic fat was negatively correlated with insulin secretion in non-diabetic subjects with impaired glucose tolerance but not in normal glucose tolerance subjects (Gerst et al. 2017). These two studies suggest that the presence of pancreatic steatosis may be relevant to the early development T2D in a specific time window of glucose intolerance, but once diabetes occurs, factors additional to pancreatic fat may explain further the decline in β cell function. In rodents, obesity induces a massive increase in pancreatic triglycerides and this accumulation was shown to precede the onset of overt diabetes, supporting the early role of pancreatic fat content in metabolic dysfunction (Gerst et al. 2019). In humans but not in rodents, free fatty acids are also stored inside endocrine cells in the form of BODIPY-positive intracellular lipid droplets, which is found at higher density in T2D than in healthy islets (Ji et al. 2019a; Tong et al. 2020). By manipulating the expression of the lipid droplet scaffold protein Perilipin 2 *(PLIN2)* in a human β cell line, the authors further show that PLIN2-derived lipid droplet formation was a protective mechanism for β cells to preserve their function and overcome lipotoxicity (Tong and Stein 2021).

During obesity and T2D, adipose tissue and adipocyte themselves are a source of inflammatory cytokines and chemokines (Gregor and Hotamisligil 2011). Pancreatic adipocytes may locally secrete such factors associated with islet inflammation. In

human pancreatic resections, a significantly higher number of $CD68^+$ macrophages were found in the islets located in proximity to adipocytes (Gerst et al. 2017). More recently, Horii and colleagues show that the number of $CD68^+$ macrophages per islet correlated significantly with fat cell area, but not with C-peptide levels or circulating C reactive protein (CRP) levels (Horii et al. 2020). Thus, ectopic pancreatic fat content, but not β cell lipid droplets, may be associated with β cell decline and local inflammation during early glucose intolerance development, preceding T2D diagnosis. Since adipocytes are also present in healthy pancreata (Gerst et al. 2017), further work is warranted to fully understand the relationship between pancreatic steatosis and metabolic disease.

7.3 Aging

Older adults are at higher risk for developing T2D due to the combined effects of increasing body fat mass, including excessive fat in visceral adipose depots, and decreasing muscle mass. Both alterations are closely linked to insulin resistance and metabolic disease. The natural aging process is also associated with a progressive decline in β cell function and proliferation and increased stress-induced β cell death (Li et al. 2019). In humans, islet lipid droplets accumulate with age, further supporting a link between fat and aging (Tong et al. 2020). Aging is also closely related to a greater inflammatory activity that may directly influence metabolic cell homeostasis, including β cells. This association may hold true for certain factors such as CRP, but not all inflammatory markers (Furman et al. 2019). In aging mice, islets show increased gene expression of *Il1b* concomitant to a decreased *Il1rn*, suggesting greater IL-1β activity in old islets. Indeed, macrophage-specific *Il1b* knockout preserved glucose-stimulated insulin secretion and β cell proliferation during aging compared to young mice (Boni-Schnetzler et al. 2021). $CD3^+$ T cells were also shown to accumulate within islets during aging, but not other immune cell types. This increase was uncoupled from body weight as similar HFD-induced weight gain in younger mice did not increase islet T cell content (Denroche et al. 2021). In the islet of aged zebrafish, signs of chronic inflammation were demonstrated, characterized by the recruitment of *Tnf*-expressing macrophages and the activation of NF-kB signaling in β cells, which then have a reduced ability to proliferate (Janjuha et al. 2018). Thus, aging may compromise β cell function and/or mass promote the development of T2D in part through increased inflammatory processes in islets.

8 In Vitro Exploration of Islet Glucolipotoxicity

Considering the technical difficulties to study human islets isolated from T2D patients, in vitro work offers an alternative to explore the impact of glucose and lipids on human β cell physiology, mimicking diabetic conditions. It has long been known that prolonged islet exposure to elevated glucose concentrations negatively

affects islet homeostasis. Original studies show that chronic high glucose concentrations impair β cell function, trigger β cell apoptosis, and induce *IL1B* gene expression in exposed human islets (Eizirik et al. 1992; Maedler et al. 2002). T2D is also associated with dyslipidemia and T2D patients have increased circulating free fatty acids (FFA) levels that may affect β cells. Early studies show that chronic exposure of islets to FFA blunts glucose-induced insulin secretion and represses β cell identity genes (Eguchi et al. 2012; Lee et al. 1994). When β cells are able to incorporate FFA into Plin2-mediated lipid droplets, β cells are protected against lipotoxicity by limiting the trafficking of FFA into mitochondria (Tong and Stein 2021). Despite all the work surrounding the effect of FFA on β cells, the concept of lipotoxicity in islets is still challenged as supra-physiological concentrations of FFA are often used in experiments and considering the current limited knowledge of β cell lipid uptake (Weir 2020). In human islets, FFA including oleate, palmitate, and stearate also induce the gene expression of pro-inflammatory cytokines such as *Il1B*, *IL6,* and *IL8*. In mouse islets, only stearate is able to trigger *Il1b* and *Cxcl1* gene expression (Boni-Schnetzler et al. 2009). Elevated glucose concentrations enhance FFA effects on human islets, suggesting that hyperglycemia and dyslipidemia may potentiate each other to favor β cell decline in T2D (Boni-Schnetzler et al. 2009; Ehses et al. 2007).

The underlying mechanisms by which elevated glucose concentrations and/or FFA impair β cell homeostasis may be through a direct effect on β cells or through the activation of islet-resident macrophages. Indeed, glucose signaling and FFA are both able to drive *Il1b* gene expression and the activation of the inflammasome NLRP3 in macrophages (Masters et al. 2010; Wen et al. 2011). Notably, both glucose and FFA-mediated effects were prevented when islets were treated with the IL-1RA, confirming that IL-1β mediates part of metabolic stress in β cells (Boni-Schnetzler et al. 2008; Boni-Schnetzler et al. 2009; Maedler et al. 2002). Since macrophages are the main source of *Il1b*/*IL1B* in mouse and human islets, macrophages are likely to be the first responder to glucolipotoxicity, amplifying the islet inflammatory response. Studies with Min6 β cell line demonstrate that palmitate can impair glucose-induced insulin secretion only in the presence of co-cultured macrophages (Eguchi et al. 2012). Therefore, interactions between β cells and macrophages are necessary to trigger islet inflammation in response to glucose and FFA and together exacerbate β cell dysfunction.

Recent work investigated various combination of high glucose levels and FFA concentrations on human islet function (Marselli et al. 2020). Both chronic exposure of islets to high glucose and palmitate impair β cell function with the maximum deleterious effect when combined. After a washout period, β cells could recover from high glucose and palmitate separately but not from the combination, confirming that glucose and FFA together exacerbate β cell decline. Transcriptomes of islets exposed to palmitate and even more so to combined palmitate and glucose (but not to glucose alone) show increased gene expression of pathways involved in lipid metabolism and inflammation while promoting β cell dedifferentiation. Importantly, these in vitro glucolipotoxicity gene expression signatures are found to have a big overlap with the transcriptomes of islets isolated from T2D patients compared to

non-diabetic individuals. Thus, the identification of shared mechanisms associated with glucolipotoxicity-induced human β cell dysfunction in vitro and T2D islet traits provides insights into T2D pathogenesis, bringing into sharp focus the importance of islet inflammation in human disease.

9 Conclusions and Perspectives

During the progression to T2D, β cell failure results from a combination of β cell function decline coupled with reduced number of β cells. Mounting evidence demonstrates that components of the innate immune system play a crucial role in regulating islet homeostasis during metabolic stress. It is accepted that insulitis is not only a feature of T1D, but also of T2D, and that macrophages, the main immune cells in islets, accumulate inside T2D islets compared to non-diabetic subjects. T2D islets are characterized by an inflammatory response that is now an accepted part of T2D etiology. Such islet inflammation may be initiated by different and synergetic mechanisms including IAPP, lipotoxicity, and glucotoxicity and is mainly orchestrated by islet-resident macrophages and their IL-1β production as illustrated in Fig. 1. Thus, islet inflammation may represent a major focus in the future therapeutic development for T2D treatment (Donath et al. 2019). Some

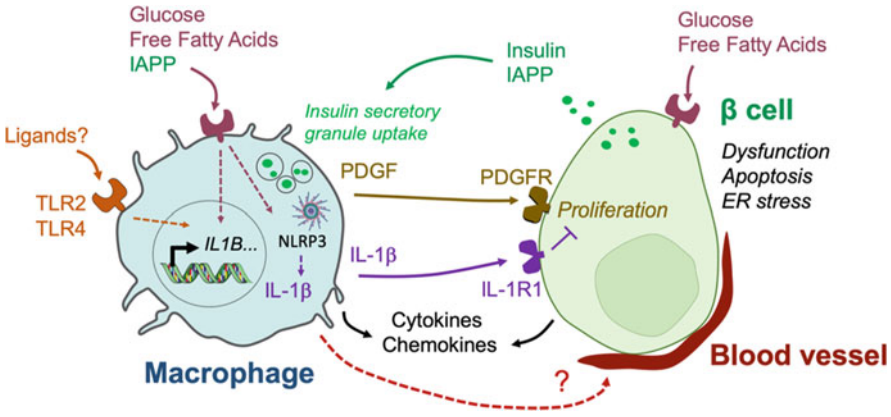

Fig. 1 Macrophage and β cell interactions in islets during obesity and T2D. During obesity-induced loss of insulin sensitivity, macrophages accumulate inside islets and contribute to β cell failure including β cell dysfunction, apoptosis, and endoplasmic reticulum (ER) stress, eventually leading to T2D. Hyperglycemia and increased circulating free fatty acids levels as well as chronic exposure to co-secreted insulin and islet amyloid polypeptide (IAPP) may activate islet macrophages and promote chemokine and cytokine production during disease progression, especially the inflammasome NLRP3-dependent IL-1β. Macrophages impair glucose-stimulated insulin secretion by both secreted factors and unclear cell–cell interactions and by internalizing β cell-derived insulin secretory granules. In parallel, islet macrophages promote β cell proliferation through the PDGFR pathway to ensure adequate β cell mass while limiting it by β cell IL-1β signaling. Macrophages also control the maintenance of islet blood vessel network via unknown mechanisms

discrepancies are reported in the literature about macrophage number and inflammatory markers in islets of T2D patients and rodent models. It may reflect differences in techniques used, disease stages, or patient heterogeneity. Ahlqvist et al. (2020) giving the recent stratification of diabetic patients who differ in etiologies and complications, islet inflammation may not affect all subgroups to the same degree and may actually be a dynamic process throughout the course of the disease spectrum. Differences may also highlight the yet limited knowledge on the nature of islet macrophages and other immune cells. Indeed, they may represent a mix of different macrophage subpopulations with distinct functions and localization within an islet at the many stages of the disease. As it was recently achieved for adipose tissue macrophages, future work may focus on single-cell genomic analyses allowing the generation of a detailed atlas of islet macrophage subsets in physiology and during metabolic disease. Identification of distinct macrophage clusters may thus give insights into their dual role in islets in obesity and T2D, during which macrophages both support islet remodeling while contributing to β cell dysfunction. As compared to other metabolic tissues, islet macrophages harbor a surprising pro-inflammatory phenotype already at steady-state, resembling barrier macrophages of the lung and the gastrointestinal tract (Ferris et al. 2017). Future studies are necessary to fully define the islet niche allowing this IL-1β-driven macrophage activation and to understand their role, cell interactions and evolution during metabolic disease. Other immune components and compartments may also be involved in islet dysfunction during T2D and need further attention including certain T cells and the pancreatic lymph nodes (Wan et al. 2018).

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Conflict of Interest Statement Nothing to declare.

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NAFLD and NASH: The Metabolically Diseased Liver

Angelo Armandi and Jörn M. Schattenberg

Contents

Abstract

Non-Alcoholic Fatty Liver Disease (NAFLD) is the most common chronic liver disease, with a global prevalence of approximately 24% in the general population. It is caused by fat accumulation in the liver secondary to insulin resistance, visceral obesity, and/or features of metabolic syndrome. A genetic susceptibility contributes to the phenotype, accounting for a more severe course of liver disease and the observed clinical variability. In fact, despite liver steatosis being considered a relatively benign entity, inflammation related to oxidative stress and lipid-derived damage may lead to non-alcoholic steatohepatitis (NASH), which constitutes the progressive disease. Accumulation of hepatic fibrosis can lead to cirrhosis and provide the environment for hepatocellular carcinoma. Obese and diabetic individuals represent a well-acknowledged high risk population. The assessment of liver fibrosis plays a crucial role in clinical setting, as liver-related

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mortality increases parallel to fibrosis stage. A liver biopsy is currently considered the reference standard for the diagnosis of NASH and the fibrosis stage, but many non-invasive tools are used with the aim of replacing histology for diagnosis and prognosis purposes. Blood based scores and liver stiffness are the most widely used and validated tools to assess liver fibrosis. Management of NAFLD resides on environmental interventions, including diet and physical activity to induce weight loss, and avoiding harmful nutrients, including fructose-sweetened beverages and high glycemic index foods, that are directly implied in liver injury. Multiple trials with investigational drugs are currently explored to treat fibrosing NASH, with promising results and it can be expected that a liver direct therapy aiming at steatohepatitis and fibrosis will become available soon.

Keywords

Liver fibrosis · Liver stiffness · Medical therapy · Metabolic syndrome · Non-alcoholic fatty liver disease · Non-alcoholic steatohepatitis · Non-invasive fibrosis tests · Obesity · Type 2 diabetes · Weight loss

1 Definition

The term NAFLD comprises a wide spectrum of pathophysiological changes in the liver. It represents the liver disease spectrum that develops with accumulation of fat in the absence of harmful alcohol use or secondary causes of hepatic steatosis. If the alteration in the hepatic compartment is restricted to fat accumulation, the term Non-Alcoholic Fatty Liver (NAFL) is used, defining any fat accumulation exceeding 5% of the hepatocyte area on liver histology. The thresholds for alcohol-induced liver disease are typically seen at 210 g/week for men and 140 g/week for women. NAFL is reversible, but predisposes to additional metabolic disease, making this condition an indicator of poor metabolic health. When relevant hepatic inflammation – characterized by lobular inflammation and ballooned hepatocytes – is present on a representative and adequate liver biopsy specimen, the inflammatory subtype non-alcoholic steatohepatitis (NASH) is diagnosed. NASH evolves from NAFL, and the hallmark is hepatocyte cell death and regeneration yielding to the activation of stellate cells that produce fibrosis aiming at tissue repair. It is through ongoing deposition of collagen and crosslinking of collagen septa, that progressive scaring leads to a nodular remodeled liver parenchyma representing liver cirrhosis. Hepatic fibrosis typically begins in the periportal space and then spreads throughout the liver lobule building bridging scars, finally cirrhosis nodules. Hepatic fibrogenesis is a dynamic process that is modeled by constant generation and degradation of collagens (Karsdal et al. 2021). At a certain point of tissue disruption with alterations in hepatic sinusoid permeability a reversion is more difficult to achieve. Once the cirrhotic stage has developed the risk of decompensation with clinically significant portal hypertension leading to ascites and variceal bleeding is real. Given the diversity of impacting factors and the individual responses to the

disease, the natural history of NAFLD is unpredictable. A majority of cases exhibit a slowly progressive course occurs and progression takes decades. Liver fibrosis may remain stable or even regress depending on the disease activity which is influenced by interacting metabolic factors. In a population that was included in a clinical trial and represents a more severe and advanced disease spectrum, approximately 20% of patients with bridging fibrosis (histological disease stage F3) evolve toward cirrhosis within 96 weeks, and 20% of cirrhotic patients develop a hepatic decompensation event (Sanyal et al. 2019; Garcia-Tsao et al. 2020; Harrison et al. 2020). In addition, hepatocellular carcinoma (HCC) can raise within the cirrhotic liver.

NAFLD remains a restrictive definition, given the difficulties around the assessment of alcohol use and the definition of disease stages based on liver histology. This is particularly important in the regulatory space, where NASH resolution or fibrosis regression is explored as surrogate endpoint for conditional drug approval. Beyond the regulatory trail space, many patients exhibit metabolically driven fat accumulation in addition to co-factors contributing to the liver disease, including harmful alcohol use or chronic viral hepatitis. Owing to this clinical reality, the term "MAFLD" (metabolic dysfunction-associated fatty liver disease) has been coined. The positive definition of the disease is a positive aspect in this ongoing academical discussion (Eslam et al. 2020). With this term, the sole presence of metabolic dysfunctions is sufficient to address liver steatosis – even in the absence of liver biopsy – as MAFLD, regardless of other co-etiologies potentially implied. This definition is a matter of debate but might support the recognition of patients and improve their management.

2 Epidemiology

The global prevalence of NAFLD mirrors the widespread burden of the metabolic syndrome (Fig. 1). The European prevalence of NAFLD is estimated at 23% of the adult population, comparable to the numbers in North America (24.1%). In the Asian populations a strong increase in the last decade has been observed, with an estimated prevalence of 27%, which is even higher in South America (30.4%) (Younossi et al. 2016; Schattenberg et al. 2021). The data on the NASH prevalence is mostly based on associated risk factors and non-invasive tests and thus in the end reflects an approximation that is dependent on obesity and T2D prevalence. Overall, the association with metabolic syndrome in NAFLD is very prevalent: the prevalence of NAFLD among obese patients reaches 95%, and NASH is present in up to 55% of these cases. Among T2DM individuals, NAFLD reaches 80% of prevalence, while NASH can develop in 80% of the total (Younossi et al. 2019a). If current prevalence rates of obesity and T2DM will remain stable, NAFLD is projected to modestly increase 30% within 2030 among European countries, but NASH is expected to increase 55%, and the proportions of advanced liver disease and liver-related mortality are modeled to double (Estes et al. 2018). The economic and social burden of the disease are high (Schattenberg et al. 2021) and there is an urgent need to define

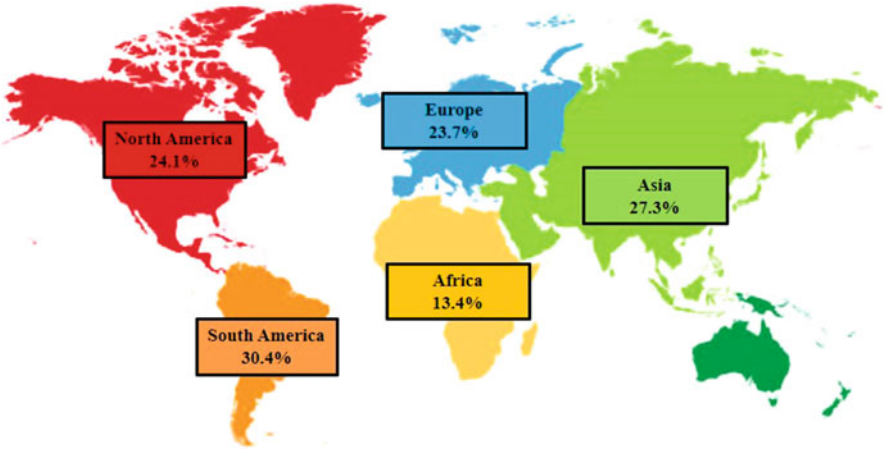

Fig. 1 Global prevalence of non-alcoholic fatty liver disease (according to Younossi et al. 2016)

models of care to link this group of patients to expert care without expanding and overwhelming health care services (Lazarus 2021).

The metabolic factors differently shape the clinical phenotype of NAFLD. In individuals without T2DM, the risk of NAFLD increases by 13–38% for every point in body mass index (BMI). On the contrary, in individuals with T2DM, the risk of developing NAFLD is independent of BMI (Golabi et al. 2019). Nonetheless, NAFLD is an independent disease process and can also develop in the absence of features of the metabolic syndrome. Multiple studies from Asian populations have reported the existence of this phenotype, often called "lean" NAFLD, but also data coming from one large American cohort assessed a 7.4% prevalence of NAFLD among lean individuals, characterized by insulin resistance, young age, and hypercholesterolemia (Younossi et al. 2018). In lean individuals, but also in patients with established metabolic co-morbidities, NAFLD is the result of a balance between environmental harms and genetic background.

According to disease stage, NAFLD impacts differently on prognosis. NAFL does not seem to significantly affect overall mortality in the available but short observational studies. Mortality is driven by cardiovascular outcomes, extrahepatic cancers (especially colon cancer), and liver disease. Conversely, bearing NASH causes an increase in liver-related mortality, which grows to 11.8 incidence rate per 1,000 persons/years, with respect to the 0.8 incidence observed in NAFL (Younossi et al. 2016). Among individuals with NASH, the prognosis in mainly linked to the fibrosis stage. All-cause mortality ratio ranges from 1.58 in mild fibrosis (Metavir stage F1) to 6.4 in severe fibrosis (Metavir stage F4), and liver-related mortality ratio ranges from 1.41 in F1 to 42.3 in F4 (Dulai et al. 2017). The progressiveness of NASH is evident when considering listing for liver transplantation, which has displayed a sharp decrease in viral-related liver disease and a dramatic increase in

NASH-related cirrhosis in the United States, ranging from about 500 liver transplants in 2002 to nearly 2000 in 2015 (Goldberg et al. 2017).

3 Pathophysiology

Insulin resistance represents a main pathophysiological basis on which tissue-specific dysfunctions develop (Fig. 2). Adipose tissue insulin resistance results in enhanced lipolysis with increased blood levels of free fatty acids (FFA), which are conveyed toward the liver and accumulated in hepatocytes as lipid droplets after re-esterification. Hepatic triglycerides are metabolized through mitochondrial β-oxidation, but excessive amount of liver fats lead to impaired lipid metabolism and augmented burden of toxic lipid intermediates, including ceramides and diacylglycerols. These lipids exert damage to cell structures (lipotoxicity) triggering an inflammatory response. Oxidative stress generated by mitochondrial exhaustion adds to cellular inflammation, inducing apoptosis. Fat intermediates themselves and inflammatory cytokines impair insulin-mediated glucose uptake, raising hepatic insulin resistance that causes enhanced gluconeogenesis and hepatic de-novo lipogenesis. In the skeletal muscle department of patients with NASH, a catabolic state and additionally increased adipose tissue derived FFA influx impairs muscle function and promotes sarcopenia.

Today, increased fructose consumption represents a major aspect of the pathophysiology in NAFLD. Fructose in sweetened beverages is metabolized in the liver to yield fat from de-novo lipogenesis contributing to the lipid-driven inflammation. The inflamed liver is a source of proinflammatory cytokines that act in synergy with

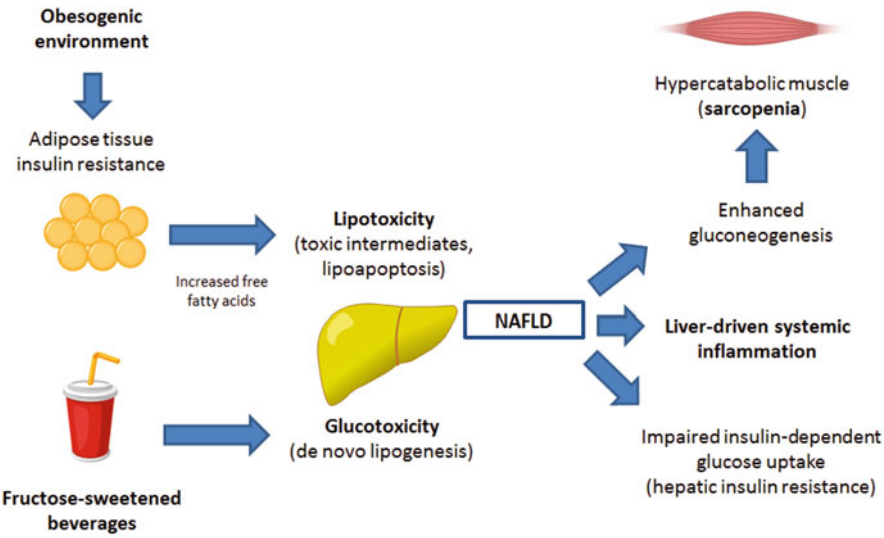

Fig. 2 Most relevant sources of liver injury in non-alcoholic fatty liver disease (NAFLD)

the inflammatory activity exerted by the visceral adipose tissue. These cytokines including interleukin-1, tumor necrosis factor-α, interleukin-6, and leptin are involved in a multi-tissue crosstalk with reciprocal implications in the affected metabolism and organ function. The interconnected pathways in patients exhibiting features of the metabolic syndrome are responsible for the complexity in the clinical phenotype. Weight gain and fat accumulation are based on dietary choices focusing on ultra-processed foods and insufficient physical inactivity. A diet rich in saturated fats, high glycemic index foods, fructose-sweetened beverages, red and processed meats, and low fiber intake are responsible for the metabolic derangements that lead to glucotoxicity and lipotoxicity. Specific eating patterns are a further burden to hepatic metabolism: attitude to snacking, rushed eating, and late eating have shown to impact on liver fat accumulation. These behavioral patterns are often underreported in clinical setting and need to be properly explored.

The typical eating pattern resembling a "Western Diet" involves high intake of carbohydrates and fat, with relative reduction in protein intake, all based on a loss of nutrients that are ultra-processed and manufactured industrially. This diet fosters abdominal fat accumulation and decreasing muscle mass and the phenotype is referred to as "sarcopenic obesity." In these patients, the loss of muscle promotes fatigue and reduces the activity. Sarcopenia occurring in individuals with metabolic syndrome significantly impacts on morbidity and worsens systemic hyperinsulinemia, hence it needs to be recognized and properly managed. Alcohol consumption represents an additive source of liver injury in this population. Small amounts of alcohol, especially red wine along with meals, are considered safe, exerting antioxidant action upon the liver from polyphenols. Nonetheless, there is no firm for NAFLD, as the beneficial effect of alcohol is counterbalanced by its calorie burden and effects on the metabolism.

Secondary causes of fatty liver and liver injury include medications that are summarized in Table 1: non-steroidal anti-inflammatory drugs (NSAIDS), oral corticosteroids, antibiotics like amoxicillin/clavulanate, immunosuppressive drugs like azathioprine, but also antiepileptic drugs (valproic acid), most antidepressants, or tamoxifen, and herbal products. In the context of the medical history these are of important aspects when assessing metabolic drivers and disease co-factors. Other liver diseases can likewise cause hepatic steatosis: hemochromatosis and Wilson Disease, but also Autoimmune Hepatitis/Cholangiopathies and hepatitis C virus infection (genotype 3). This highlights the importance of a complete laboratory evaluation to explore differential diagnosis. During laboratory evaluation, elevated ferritin levels are frequently observed and occur in about 30% of patients with NAFLD in the absence of iron accumulation. As acute phase protein, ferritin is upregulated during systemic inflammation, and hence it increases in NAFLD which constitutes a chronic, low-grade inflammation promoted by metabolic syndrome, but can also mirror an increased intrahepatic inflammatory activity. Moreover, some extrahepatic diseases may affect the liver. The most relevant are celiac disease and thyroid disease. In particular, elevated liver enzymes observed in celiac disease is the liver expression of gut malabsorption. As thyroid hormones are directly involved in

Table 1 Causes of liver steatosis

Primary steatosis
Non-alcoholic fatty liver disease[a]
Alcoholic fatty liver disease[b]
Secondary steatosis
Drug-induced liver injury[c]
Hepatitis C Virus infection (genotype 3)
Wilson disease (microvescicular)
Alpha-1-antitrypsin deficiency
A/hypo-beta lipoproteinemia
Starvation
Parenteral nutrition
Hereditary errors of metabolism (e.g., lysosomal acid lipase deficiency)
Hypopituitarism
Hypothyroidism
Celiac disease

[a]Associated to features of Metabolic Syndrome, especially obesity (Body Mass Index >30 kg/m^2) or Type 2 Diabetes Mellitus (blood glucose level $\geq$126 mg/dl or treated)
Other metabolic co-factors included in the metabolic syndrome:
- Waist circumference $\geq$94 cm for men and $\geq$80 cm for women
- Arterial blood pressure $\geq$130/85 mmHg, or treated Impaired Fasting Glucose (blood glucose level $\geq$100 mg/dl)
- Serum triglycerides $<$150 mg/dl
- High Density Cholesterol $<$40 mg/dl for men and $<$50 mg/dl for women

[b]Alcohol intake $>$210 g/week for men and $>$140 g/day for women
[c]Non-Steroid Anti-Inflammatory Drugs, systemic corticosteroids, tamoxifen, methotrexate, tetracycline, estrogens, valproic acids, amoxicillin/clavulanate, azathioprine, herbal products

lipid metabolism, a reduced thyroid activity may result in hypercholesterolemia and liver fat accumulation.

Genome Wide Association Studies (GWAS) have highlighted a number of genetic traits (mainly single nucleotide polymorphisms) that convey a susceptibility to liver disease, irrespective of the etiology. PNPLA3 (Patatin-like phospholipase domain-containing protein 3), TM6SF2 (Transmembrane 6 Superfamily Member 2), and MBOAT7 (Membrane Bound O-Acyltransferase Domain Containing 7) are the main genes associated with a worse phenotype of liver disease, being involved in the intrahepatic lipid metabolism and which polymorphisms confer a less capability to export lipids toward the periphery, favoring lipid-driven injury.

4 Diagnostics

According to European guidelines, patients with features of metabolic syndrome and in particular T2DM should be screened for NAFLD (EASL 2016, 2021). Similarly, evidence of liver steatosis demands the search for metabolic risk factors. Liver biochemistry may display no alterations, or only mild elevations of transaminases,

often accompanied by gamma-glutamyl transferase (GGT) increase. In particular, elevation of GGT is highly suspicious for T2DM and/or cardiovascular risk factors.

Individuals affected by NAFLD are most often unaware of this condition. They present with an incidental finding of liver steatosis at ultrasound and/or unexplained altered liver biochemistry. The diagnosis of NAFLD requires the exclusion of other known causes of liver disease, along with the presence of metabolic risk factors. Physical semeiotics of chronic liver disease is poor, but a careful interview on medical history and voluptuary habits provides essential information on the etiology. The investigation of metabolic affections of metabolic syndrome requires a comprehensive evaluation for hypertriglyceridemia, hypercholesterolemia with decreased high-density lipoproteins (HDL), increased blood pressure, increased waist circumference, increased body weight by BMI, impaired fasting glycemia or frank T2DM are the main hallmarks of metabolic-driven damage. In addition, morbidly obese individuals often suffer from obstructive sleep apnea syndrome (OSAS). The poor quality of night sleep leads to a daily sleepiness, increased perception of fatigue and inactivity, promoting a sedentary lifestyle and worsening liver disease.

Given the central role of insulin resistance in this liver disease, all patients with suspected NAFLD should undergo evaluation of blood glucose and insulin levels. The Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) is a useful, first-line tool to assess insulin resistance that combines both insulin and glucose values in a formula. Values above 2.5 are highly suspicious for insulin resistance. Abdominal ultrasound is part of the routine exploration. Steatosis and an inhomogeneous echogenic texture of the liver parenchyma are common findings. Ultrasound plays a central role in the evaluation of focal liver lesions and features of portal hypertension. In patients with chronic liver disease, however, it does not provide useful information, because of the inaccuracy to quantify steatosis, and the impossibility to stage fibrosis. From a hepatologist's perspective, the degree of hepatic steatosis and fibrosis stage are important. When NAFLD is present, identification of NASH, in particular fibrosing NASH, is the most relevant aspect. Currently, the accepted reference to diagnose NASH and stage hepatic fibrosis is liver biopsy. Considering the invasiveness of the procedure, it is important to keep in mind that a minimum requirements to allow for histological assessment are necessary. Liver tissue should be non-fragmented, of adequate length and contain a number of portal tracts for a reliable diagnosis. The semi-quantitative Clinical CRN scale (Brunt Score) is currently used to define NASH. A scoring system quantifies steatosis (0–3), lobular inflammation (0–2), and ballooning (0–2) to the Non-Alcoholic Steatohepatitis (NAS) score, which value equal or above 4, including all the above features, defines NASH. Metavir score for fibrosis staging (0–4) is applied. More recently, another score system, the Steatosis Activity Fibrosis (SAF) score has been proposed, with the aim of separating steatosis from the inflammatory features, providing more reliable results (Bedossa et al. 2012). The costs, its limited accessibility, and the complication rate that can occur in up to 1% of cases are limiting its broad use. More importantly, inherent sampling variability and inter-observer variations between pathologists have to be considered. These limitations have strongly supported the development of non-invasive fibrosis tools (Table 2) that

Table 2 Most widely validated non-invasive tools for the assessment of liver steatosis and fibrosis

Type of the exam	Strengths	Limitations
Abdominal ultrasound	• High availability • Optimal for focal liver lesions, biliary tree, and features of portal hypertension	• Operator dependent • Inaccurate for steatosis <20% • Qualitative assessment of steatosis
Transient elastography	• High specificity for significant and advanced fibrosis • High reproducibility	• Operator dependent • High costs and limited availability • Reduced accuracy in obese individuals
Controlled attenuation parameter	• Quantification of steatosis grades • High reproducibility	• Operator dependent • High costs and limited availability • Reduced accuracy in obese individuals
NAFLD fibrosis score	• Inexpensive and easy to use • Good accuracy to rule out advanced fibrosis	• Low sensitivity • Low accuracy for early fibrosis stage • 30% indeterminate results
Fibrosis-4 score	• Inexpensive and easy to use • Good accuracy to rule out advanced fibrosis	• Low sensitivity • Low accuracy for early fibrosis stage • 30% indeterminate results

are now used routinely to assess biochemical and anthropometric parameters as surrogates of liver fibrosis. By applying these tests sequentially, the need for liver biopsy can be reduced. The NAFLD Fibrosis Score (NFS) and Fibrosis-4 (FIB-4) score are the most widely used and validated non-invasive tools to assess fibrosis. Overall, they provide higher negative predictive values and lower positive predictive values, being more accurate in excluding advanced fibrosis (F3 or F4). They show less accuracy for initial fibrosis stages (F0 through F2) and are burdened by about 30% of undetermined results, with differences across studies (Alqahtani and Schattenberg 2021). The strength of these NITs is the board availability in most setting and the low costs.

A more accurate imaging-based tools to evaluate liver fibrosis is transient elastography (TE) using Fibroscan. TE uses low frequency vibrations to explore liver stiffness. It provides more reliable results for higher stages of fibrosis and has overall high specificity and lower sensitivity to detect advanced fibrosis. However, TE has limited accessibility, higher costs, and some inter-operator variability. One additional feature that is simultaneously calculated along the stiffness examination is Controlled Attenuated Parameter (CAP). CAP quantifies hepatic steatosis in a continuous measure, providing a useful assessment that is superior to US and guides clinical decision making. An algorithm for liver disease-related diagnostic workup in

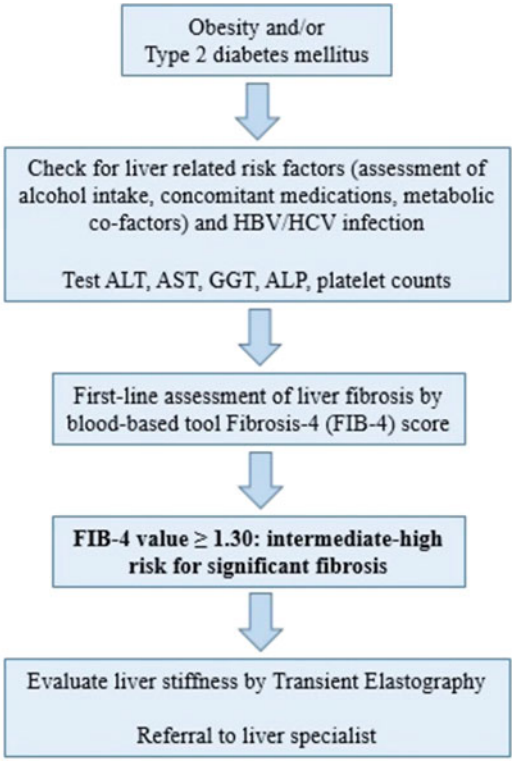

Fig. 3 Diagnostic workup for the assessment of liver disease in patients with obesity and/or type 2 diabetes

obese/diabetic individuals is depicted in Fig. 3. Today, magnetic-resonance (MR) based techniques are being evaluated to assess both steatosis (MR-Proton Density Fat Fraction [MR-PDFF]) and fibrosis (MR Elastography [MRE]). Preliminary results coming from clinical trials have shown the highest accuracy among non-invasive techniques, yet burdened by high costs, that limit their applicability to experimental purposes. MR-PDFF correlates well with histology, has the ability to discriminate liver fat from inflammation, edema, and iron accumulation, and is superior to CAP in fat quantification. MRE has shown highest accuracy to detect advanced stages of fibrosis and is superior to TE in the assessment of early stages of fibrosis (Loomba 2018).

Image-based investigations are accurate, yet static evaluations. Alternative approaches to detect liver fibrosis have risen from experimental studies on liver fibrogenesis, which is the process that leads to scar deposition, and fibrolysis, that is the process of scar removal. Deposition of matricellular matrix, mainly constituted by collagens and integrins, is a highly dynamic process, continuously shaped by environmental harms, intrahepatic signaling, and host responses. Proteomic studies have identified several collagen fragments potentially linked to either fibrogenesis or fibrolysis processes (Schuppan et al. 2018). This approach seems promising, because of the possibility to gain further information of the actively ongoing disease process,

displaying the ability to identify individuals at higher risk for disease progression. Moreover, quantification of blood matricellular peptides can mirror the activity of the underlying disease process, discriminate between diverse fibrosis stage, and predict response to drug treatment.

Pro-C3 (N-terminal propeptide of type III collagen) is a fibrogenesis marker that has shown the best potential to detect significant fibrosis and disease progression. In contrast to other tests, it is linked directly to the building of collagens in the hepatic compartment and thus resembles a potentially dynamic marker to assess patients and the degree of fibrogenesis. In healthy controls, Pro-C3 levels are in the range of 6.1–14.7 ng/ml, increasing twofold in the advanced disease stages of NAFLD (Erhardtsen et al. 2021). Another recent approach is based on the Enhanced Liver Fibrosis (ELF) test, which is a combination of three direct serum markers of fibrosis: hyaluronic acid, TIMP1 (Tissue inhibitor matrix metalloproteinase 1), and PIIMP (procollagen III amino-terminal peptide). The combined quantification of these markers, which cover a different role in the fibrosis process, has been established to predict unfavorable outcomes in patients that have had a liver biopsy showing advanced liver disease (Vali et al. 2020). Future findings will assess the role of this novel approach in the stratification and prognostication of patients with fibrosing NASH.

Ultimately, the expanding field of artificial intelligence (AI) in healthcare has led to the development of Machine Learning (ML) tools to analyze huge amounts of data and fulfill unmet needs in clinical practice. Individualizing patients with NASH among NAFLD individuals combining clinical features and non-invasive scores; improving the appropriateness of histological scores of the disease; gaining objective information from image-based techniques to avoid inter-operator variability are some examples of the fields where AI is undergoing a progressive validation in larger cohorts and will be a strong tool in clinical setting (Dinani et al. 2021; Docherty et al. 2021; Noureddin 2021).

5 Management

5.1 Lifestyle

As a greater proportion of patients with NAFLD are obese, a careful management of weight loss is required (Table 3). This is not easily achieved in clinical practice, due to underreporting and lack of compliance. A weight loss of 5–10% is sufficient to obtain a resolution of steatohepatitis and fibrosis regression. The quality of diet and type of nutrients have received a lot of attention (Armandi and Schattenberg 2021). Centrally to all attempts is lasting adherence to the weight loss goals. In addition, correction of food misbehaviors such as attitude to snacking and rushed eating is part of the interventional program. High intake of ultra-processed foods, in particular meat, saturated fats, and high glycemic index foods as well as sweetened beverages is critically involved in the pathogenesis of NAFLD. Since alcohol consumption in patients with NAFLD has not been shown to be cardio-protective, abstinence can be

Table 3 Non-pharmacological management of non-alcoholic fatty liver disease

Weight loss	Calorie restriction to about 1,000 kcal/day Weight loss of at least 8% of the initial is recommended for overweight/obese individuals. A tailored approach to improve compliance and long-term maintenance of weight loss is suggested
Physical activity	Moderate aerobic physical activity (200 min/week divided in 3–5 sessions of walking or biking) improves weight loss and insulin sensitivity
Alcohol intake	Alcohol intake <210 g/week for men and <140 g/week for women, consumed with meals, is considered safe in non-cirrhotic patients
Coffee drinking	No liver-related restrictions
Behavioral aspects	Night eating, binge eating, snacking, rushed eating, craving for carbohydrates, emotional eating are behavioral patterns that worsen liver disease and are often underreported. Modifications of eating behavior are recommended
Macronutrient composition	Avoid high glycemic index foods, limit consumption of red and processed meats and saturated fats. Encourage consumption of fibers and mono and polyunsaturated fats
Sweetened beverages	Avoiding fructose- and sucrose-sweetened beverages is mandatory

recommended. On the other hand, patients reporting significant loss in quality of life with abstinence, less than moderate consumption can be upheld. At current the available data does show harmful effects at levels that are less than moderate consumption. Patients with cirrhosis (and most likely advanced fibrosis) should be abstinent.

For a Mediterranean-type diet most data has been generated and improvement of insulin resistance, to a lesser extent, improvement of NAFLD has been shown. However, geographical and culture differences, together with issues related to food costs and accessibility, limit the widespread adoption of Mediterranean Diet. An individualized approach and patient empowerment seem to be critical. Low glycemic index foods have to be encouraged, given the central role of insulin sensitivity in the pathogenesis of NAFLD. Mono- and polyunsaturated fats and high assumption of fibers are recommended to obtain a better control of blood lipids and proper composition of gut microbiota, respectively. Physical activity is part of the interventional strategy aiming at weight loss and improvement of insulin sensitivity. Aerobic exercise is suggested, like walking or cycling, for at least 60 min 5 days per week.

5.2 Pharmacotherapy

Currently, no approved drug treatment for NAFLD is available and its management relies mostly on the correction of metabolic risk factors. As NAFLD is part of a multi-systemic disease, a multidisciplinary approach is commonly required. In particular, a proper control of blood pressure and normalization of dyslipidemia and T2D are crucial to arrest liver disease progression.

A number of studies have been conducted on the most widely used diabetes medications and there is potential to exert benefit in NAFLD. For metformin, no benefit beyond its prominent role in glycemic control has been shown in terms of liver histology. On the other hand, the safety data of metformin in patients with NAFLD is very strongly supporting its use in this population, in particular as association studies have indicated that the risk of HCC is lower (Zhang et al. 2013). Peroxisome proliferator-activated receptors (PPAR) are a family of nuclear receptors that exert multiple regulatory effect in lipid and glucose metabolism. The PPAR α and δ agonist pioglitazone has been studied in several trials in adults and children – both with and without diabetes (Belfort et al. 2006; Sanyal et al. 2010; Cusi et al. 2016). Lanifibranor, a pan-PPAR agonist, is currently being tested in a phase 3 study as treatment for fibrosing NASH after promising evidence of improvement in steatosis and no worsening in fibrosis in a previous phase 2 study (Francque et al. 2020). A positive impact on steatohepatitis was observed in patients with T2DM. Conversely, the use of Vitamin E at high dosage of 800 IU twice daily showed a benefit in patients without T2DM. More recently, novel approaches with investigational drug treatments for NASH mirror the multifaceted shape of the disease. The effect of glucagon-like peptide 1 (GLP-1) agonists liraglutide and semaglutide in patients with fibrosing NASH reported a significant improvement in steatohepatitis without affecting fibrosis (Armstrong et al. 2016; Newsome et al. 2021). Other medications approved in the T2DM populations like sodium-glucose cotransporter-2 (SGLT2) inhibitors have shown to improve liver fat content evaluated by MRI-PDFF (Kuchay et al. 2018). Anti-fibrotic strategies have been developed based on evidence that FXR-agonists are capable to reduce inflammation and stellate cell activation. Obeticholic acid (OCA) is a semi-synthetic biliary acid analogue approved as second-line treatment for primary biliary cholangitis. OCA is an agonist of nuclear Farnesoid X Receptor with multiple effects in both biliary and hepatocyte metabolism. In a pivotal phase 3 trial OCA has been the first drug to show fibrosis regression on liver histology in NASH that exceeded the placebo effect albeit all shortcomings a histological endpoint poses (Younossi et al. 2019b). Given the role thyroid hormones play on lipid metabolism, the hepatocyte thyroid hormone pathways have also been targeted. Resmetirom is a liver-selective thyroid hormone receptor beta agonist that has shown to improve MRI-based liver fat content in a phase 2 trial (Harrison et al. 2019) and is currently tested in a large phase 3 trial, being one of the most promising drugs for NASH. Considering the multiple mechanisms that are involved in the pathophysiology of NASH, it seems likely that a combination approach will be required to tackle NASH.

Bariatric surgery is an alternative approach to treat morbid obesity associated NAFLD. Currently, the most practiced technique is sleeve gastrectomy, which implies a large reduction in gastric volume to reduce appetite. Weight loss is quickly achieved right after surgery, with a stabilization following the second year. The associated liver disease displays a persistent amelioration after 5 years following surgery (Lassailly et al. 2020). Endoscopic approaches are under evaluation, with promising results. Intragastric balloons or endoscopic sleeve gastroplasty to reduce appetite, or duodenal mucosal resurfacing to improve glucose homeostasis has been

used in obese and T2DM cohorts and might be usefully implied in NAFLD populations (Salomone et al. 2020; Bazerbachi et al. 2021). Albeit these promising data, NAFLD is not an indication that bariatric surgery is being performed for and most studies have explored liver histology only in patients that are being treated for obesity, not underlying liver disease.

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Diabetic Kidney Disease: From Pathogenesis to Novel Treatment Possibilities

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Abstract

One of the microvascular complications of diabetes is diabetic kidney disease (DKD), often leading to end stage renal disease (ESRD) in which patients require costly dialysis or transplantation. The silent onset and irreversible progression of DKD are characterized by a steady decline of the estimated glomerular filtration rate, with or without concomitant albuminuria. The diabetic milieu allows the complex pathophysiology of DKD to enter a vicious cycle by inducing the synthesis of excessive amounts of reactive oxygen species (ROS) causing oxidative stress, inflammation, and fibrosis. As no cure is available, intensive research is required to develop novel treatments possibilities. This chapter provides an overview of the important pathomechanisms identified in diabetic kidney disease, the currently established therapies, as well as recently developed novel therapeutic strategies in DKD.

Keywords

Diabetic kidney disease · Diabetic nephropathy · Fibrosis · Inflammation · NADPH oxidase · Oxidative stress

1 Introduction

Diabetic kidney disease (DKD), also referred to as diabetic nephropathy, is a chronic disease of the kidney and one of the most prevalent microvascular complications of diabetes mellitus. Apart from microvascular complications, diabetic patients with and without DKD encounter also increased risk of cardiovascular morbidity and premature mortality (Groop et al. 2009; Penno et al. 2021; McCullough et al. 2007). Of all diabetic patients, up to 30–45% develop DKD, often progressing to end stage renal disease (ESRD). Patients suffering from ESRD become imperatively dependent on dialysis or kidney transplantation, and approximately 45% of all ESRD cases are related to diabetes (Schiffer and Friedrich-Persson 2017; Ostergaard et al. 2020). Although the absence of pathognomonic symptoms impedes early detection of chronic kidney disease (CKD), diagnosis of DKD is based on the presence of

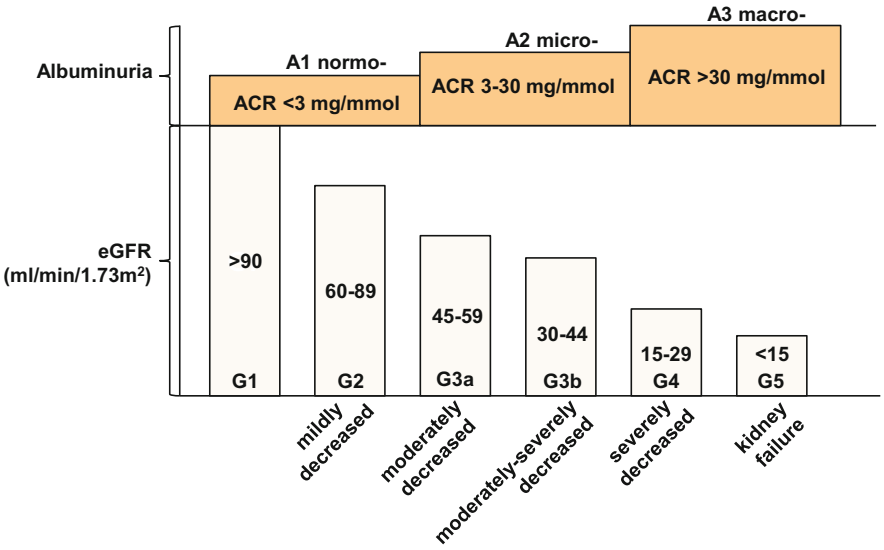

Fig. 1 Definition of diabetic kidney disease. Normoalbuminuria is defined by albumin/creatinine ratios (ACR) of <3 mg/mmol (A1), followed by microalbuminuria, which ranges between 3 and 30 mg/mmol (A2) and macroalbuminuria with an ACR >30 mg/mmol (A3). Kidney function is assessed by eGFR and progresses from normal/healthy (G1, >90 ml/min/1.73 m^2) with a steady decline (G2–G4) ultimately resulting in kidney failure (G5, eGFR < 15 ml/min/1.73 m^2)

micro- or macroalbuminuria with a progressive decline in renal function assessed by the estimated glomerular filtration rate (eGFR) (Fig. 1) (Dagogo-Jack 2021).

1.1 Definition

One characteristic of DKD is albuminuria, which is usually assessed by the albumin-creatinine-ratio (ACR) in a spot urine sample. An ACR of <3 mg/mmol is considered normal (A1), followed by a moderate increase to 3–30 mg/mmol known as microalbuminuria (A2), and a severe increase to >300 mg/mmol, also called macroalbuminuria (A3) (Fig. 1) (Dagogo-Jack 2021). In addition to the ACR, the eGFR is another important clinical indicator of DKD and the decline of eGFR can occur with or without concomitant albuminuria (Tsalamandris et al. 1994). Moreover, glomerular hyperfiltration, that is increased eGFR, is considered as an indicator for the onset of DKD, particularly in type 1 diabetes (Dagogo-Jack 2021). Healthy kidneys show values of ≥90 mL/min/1.73 m^2, followed by an eGFR of 60–89 mL/min/1.73 m^2 considered as mildly decreased, an eGFR of 45–59 mL/min/1.73 m^2 as mildly to moderately decreased, an eGFR of 30–44 mL/min/1.73 m^2 as moderately to severely decreased, an eGFR of 15–29 mL/min/1.73 m^2 as severely decreased, and finally an eGFR of <15 mL/min/1.73 m^2 considered as kidney failure (Fig. 1) (Dagogo-Jack 2021).

1.2 Diagnosis

Early diagnosis is pivotal to mitigate and delay the progression of DKD. Patients with type 1 diabetes should be monitored for ACR and eGFR annually 5 years after diagnosis, while patients with type 2 diabetes should be monitored annually directly when diagnosed with type 2 diabetes as the exact onset of kidney disease is often unclear (Dagogo-Jack 2021). Noteworthy, patients with prediabetes have also been reported to show impaired function of the kidney and/or albuminuria (Plantinga et al. 2010). The mainstay of treatment of DKD includes control of blood glucose. Although these strategies enable a delay of DKD progression, none of these approaches is able to cure kidney disease.

2 Pathogenesis of DKD

The renal physiology is maintained mainly by four cell types involving glomerular endothelial cells, podocytes, mesangial cells, and tubular cells. The well-orchestrated interaction of these cells portrays high complexity of mutual influences making the pathological origin of DKD a challenge to investigate. In the diabetic milieu, several factors including hyperglycemia and associated glucose toxicity, advanced glycation end products (AGEs), growth factors, hemodynamic and hormonal changes contribute to the harmful generation of reactive oxygen species (ROS), which in turn result in renal inflammation and fibrosis (Fig. 2) (Jha et al. 2016a). These alterations cause pathologic functional and structural abnormalities including glomerular basement membrane (GBM) thickening, podocyte loss, mesangial expansion, and eventually glomerulo- and tubulointerstitial sclerosis (Steffes et al. 1992; Kanwar et al. 2008). The glomerular filtration barrier is composed of three layers consisting of fenestrated glomerular endothelial cells, podocytes, and the GBM, which is established by both cell types (Lassen and Daehn 2020). The physiological GBM itself consists mainly of an anionic charged heparan sulfate barrier, followed by a layer of collagen IV, laminin, fibronectin, entactin and proteoglycans called the lamina densa, and another layer of heparan sulfates (Mason and Wahab 2003). These layers ensure passing of selected small molecules while preventing larger molecules from entering the Bowman's space such as albumin (Lassen and Daehn 2020; Lin and Susztak 2016). Under diabetic conditions, the interplay of the filtration barrier components is disturbed, potentially causing albuminuria. Glomerular endothelial cells allow glucose entry independently of insulin, making them specifically vulnerable to the direct exposure of blood glucose levels. In order to metabolize excess intracellular glucose levels due to hyperglycemia, glomerular endothelial cells undergo a phenotypic switch by turning on the polyol-, hexosamine-, AGE/RAGE-, and the PKC pathway, causing the generation of increasing amounts of ROS, which in turn can lead to endothelial nitric oxide synthase uncoupling and reduced nitric oxide bioavailability (Lassen and Daehn 2020; Reidy et al. 2014; Jourde-Chiche et al. 2019). The alterations in their physiology result in the degradation of the heparan sulfate layer, thereby

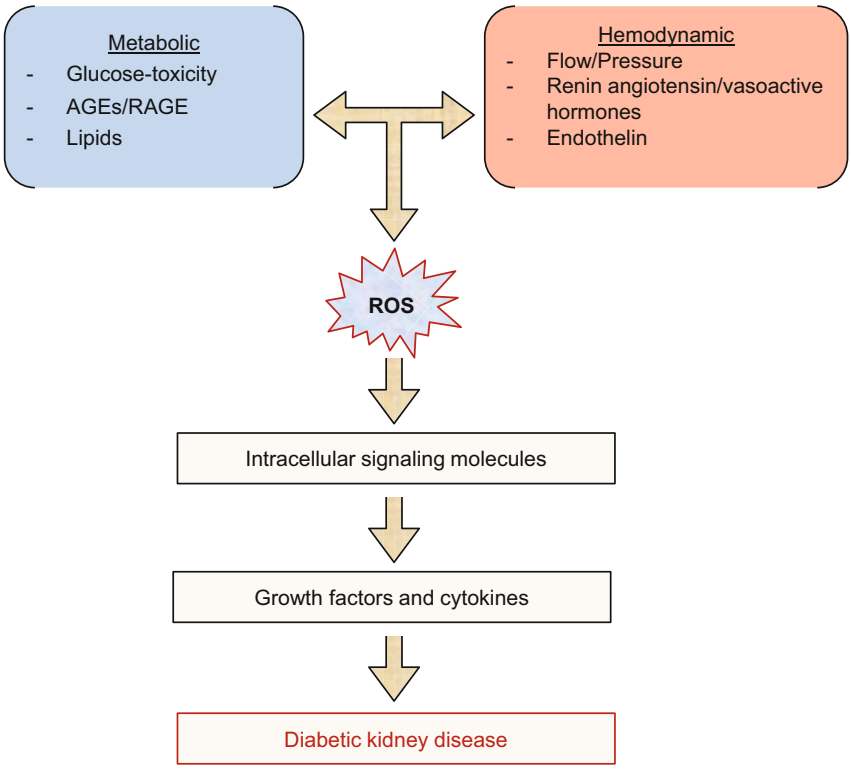

Fig. 2 Metabolic and hemodynamic changes in the diabetic milieu. The diabetic condition leads to altered cellular glucose metabolism resulting in activation of unfavorable signaling pathways. The activation of hemodynamic and metabolic pathways is associated with excessive ROS production, which leads to increased intracellular signaling ensuing the synthesis and recruitment of growth factors and cytokines, leading to a vicious cycle of inflammation and fibrosis in the diabetic kidney

affecting GBM integrity (An et al. 2018; van den Hoven et al. 2009). Maintaining the glomerular filtration function is also dependent on a crosstalk between glomerular endothelial cells and podocytes as endothelial dysfunction impairs podocytes and vice versa (Lassen and Daehn 2020; Cassis et al. 2019). Some mediators of the crosstalk involve vascular endothelial growth factor A (VEGF-A), angiopoietins, endothelin-1, activated protein C, or transforming growth factor-β (TGF-β), which stimulate podocytes to synthesize extracellular matrix (ECM) proteins (reviewed in Lassen and Daehn 2020; Marshall 2016). In diabetes, GBM thickening can also be promoted by the action of AGEs, which in the kidney does not only lead to ROS, glomerular hypertrophy, inflammation and renal fibrosis but also to podocyte injury and apoptosis (Chuang et al. 2007). Podocyte injury leads to cytoskeletal reshaping, a process termed foot process effacement, and weakens the structure of the GBM (Lin and Susztak 2016; Mundel and Shankland 2002). There is contradicting data concerning foot process effacement enabling podocytes to detach from GBM (Lin

and Susztak 2016). Loss of podocytes by apoptosis, or potentially as a result of GBM detachment, can also occur by epithelial-mesenchymal transition (EMT) induced in hyperglycemia by activating TGF-β/Smad, Wnt/β-catenin, integrins/integrin-linked kinase, MAPK, Jagged/Notch, and NFκB signaling pathways. Tubular cells undergo also EMT and together with endothelial-mesenchymal transition (EndoMT), they contribute to the formation of myofibroblasts known to produce ECM proteins and tubulointerstitial fibrosis (Loeffler and Wolf 2015). Mesangial cell injury induced by hyperglycemia additionally enhances the deposition of ECM proteins into the mesangium (Loeffler and Wolf 2015; Tung et al. 2018). The accumulation of ECM proteins, predominantly of different collagens, fibronectin and laminin, leads to scarring of the renal tissue, an important process in the progression of diabetic nephropathy called glomerulosclerosis and interstitial fibrosis, resulting in kidney failure (Loeffler and Wolf 2015; Qian et al. 2008).

3 Current Therapies for DKD

3.1 Lifestyle Changes

The general advice for patients with DKD is to maintain a healthy weight, the cessation of smoking, regular physical activity, and a reduction in dietary sodium. A reduction in dietary sodium to <2,300 mg/day can improve blood pressure control and decrease the risk for cardiovascular disease in patients with CKD (Mills et al. 2016). The advice regarding protein intake suggests a protein intake of 0.6–0.8 g/kg/weight/daily (American Diabetes Association 2021). At this level, a modest protein intake has shown to slow the deterioration of renal function. A higher protein intake is associated with glomerular hyperfiltration, an increase in albuminuria and worsening of renal function. However, a further reduction in protein intake to <0.8 g/kg/weight/daily does not further improve renal function and also carries the risk of malnutrition (Murray et al. 2018). Furthermore, many patients with reduced eGFR have elevated potassium levels due to a reduced excretion of potassium, thus diet advice needs to be adjusted on an individual basis for those patients (Kidney Disease: Improving Global Outcomes Diabetes Work Group 2020).

3.2 Blood Glucose and Blood Pressure Control

Blood glucose and blood pressure control remain the mainstay of risk factor control in the treatment of DKD. Many studies have shown that the optimization of blood pressure and glucose control retards the progression of DKD (UK Prospective Diabetes Study (UKPDS) Group 1998; Holman et al. 2008; ADVANCE Collaborative Group and Patel 2008; DCCT/EDIC Research Group and de Boer 2011; Lewis et al. 1993). However, studies by Fioretto et al. have shown that normalization of blood glucose in type 1 diabetic patients who received a pancreas transplant requires 5–10 years to reverse glomerular and tubulointerstitial changes with arteriolar

hyalinosis remaining unchanged even after 10 years of normoglycemia (Fioretto et al. 1998).

3.2.1 Blood Glucose

There is overwhelming evidence that a reduction in blood glucose is associated with a reduction in microvascular complications, in particular nephropathy. Blood glucose control is usually achieved with oral drugs in type 2 diabetic patients, whereas type 1 diabetic patients require insulin. In terms of oral drugs, metformin, an AMP-activated protein kinase (AMPK) activator, has many beneficial metabolic actions and is recommended as a first-line agent. However, metformin needs to be dose-adjusted in CKD (eGFR <45 mL/min) and is contraindicated if eGFR is <30 mL/min due to the increased risk of lactic acidosis. The target of an HbA1c of 7% or lower has shown to reduce the risk of development and progression of DKD in type 1 and type 2 diabetes (UK Prospective Diabetes Study (UKPDS) Group 1998; Holman et al. 2008; ADVANCE Collaborative Group and Patel 2008; DCCT/EDIC Research Group and de Boer 2011; Diabetes Control and Complications Trial Research Group and Nathan 1993; The Writing Team for the Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications Research Group and Steffes 2003). In the DCCT trial, patients with type 1 diabetes were allocated to an intensive and a conventional treatment arm and were followed for 6.5 years. The patients in the intensively treated group showed a reduction of albuminuria development of 34% and for macroalbuminuria of 56% (The Diabetes Control and Complications (DCCT) Research Group 1995). The follow-up study (EDIC), which evaluated patients after a median follow-up of 22 years after HbA1c values had converged to approximately 8%, showed a sustained risk reduction of 50% for deterioration of eGFR in the previously intensively treated group (DCCT/EDIC Research Group and de Boer 2011). It has been suggested that this observation, also entitled "metabolic memory", may be at least in part mediated by epigenetic mechanisms (Reddy et al. 2015). In type 2 diabetes, the UKPDS study investigated intensive glycemic control with an HbA1c of 7% versus 7.9% in the conventional treatment group (American Diabetes Association 2000). Intensive glucose control decreased albuminuria risk and had a 37% reduction in the renal endpoint of doubling of serum creatinine (Stratton et al. 2000). Similar to the findings in type 1 diabetes, after 10 years follow-up and with glycemic convergence, benefits persisted on renal and other microvascular outcomes in type 2 diabetes (Holman et al. 2008). These landmark trials show that a glycemic control with an HbA1c of approximately 7% is associated with a significantly reduced risk of DKD. In established CKD, optimization of glycemic control delays the further decline in renal function. Similarly, prospective randomized studies have shown that intensive glycemic control can also delay onset and progression of albuminuria. It should be noted that CKD can increase the half-life of some medications due to reduced renal excretion, which can lead to life-threatening hypoglycemic events. Thus, HbA1c targets need to be adjusted as part of a personalized medicine approach, taking each patient's individual risk factors and concomitant diseases into account.

Table 1 Renal outcomes in studies with SGLT-2 inhibitors or GLP-1 receptor agonists in type 2 diabetes

Study	SGLT2-inhibitors	Renal outcome	HR (95% CI)
CANVAS program (Neal et al. 2017)	Canagliflozin	>40% eGFR loss, ESRD, renal death	0.86 (0.75–0.97)
CREDENCE (Perkovic et al. 2019)	Canagliflozin	Doubling creatinine, ESRD, renal, or CV death	0.70 (0.59–0.82)
DECLARE-TIMI58 (Wiviott et al. 2018)	Dapagliflozin	>40% eGFR loss, ESRD, renal death	0.93 (0.84–1.03)
DAPA-CKD (Heerspink et al. 2021)	Dapagliflozin	>50% decline in eGFR, ESRD, renal or CV death	0.61 (0.51–0.72)
EMPAREG-OUTCOME (Wanner et al. 2016)	Empagliflozin	Doubling creatinine, ESRD, renal death	0.61 (0.53–0.70)
SCORED (Bhatt et al. 2021)	Sotagliflozin	>50% eGFR loss, ESRD	0.61 (0.51–0.72
VERTIS-CV (Cannon et al. 2020)	Ertugliflozin	Doubling creatinine, ESRD, renal death	0.97 (0.85–1.11)
	GLP-1 receptor agonists		
EXSCEL (Holman et al. 2017)	Exenatide	>40% eGFR loss, ESRD, renal death	0.85 (0.74–0.98)
LEADER (Marso et al. 2016a)	Liraglutide	Doubling creatinine, ESRD	0.78 (0.67–0.92)
REWIND (Gerstein et al. 2019)	Dulaglutide	>30% eGFR loss, ESRD, renal death	0.85 (0.77–0.93)

SGLT-2 Inhibitors

Initially, sodium glucose co-transporter 2 (SGLT-2) inhibitors were developed to reduce hyperglycemia, but recent studies have demonstrated additional renoprotective and cardiovascular effects (Table 1) (Neal et al. 2017; Perkovic et al. 2019; Wiviott et al. 2018; Heerspink et al. 2021; Wanner et al. 2016; Bhatt et al. 2021; Cannon et al. 2020). At least part of this effect is independent of glucose control and occurs down to a kidney function of 25 mL/min/1.73 m^2. SGLT-2 inhibitors block the glucose sodium co-transporter in the proximal tubule, thus leading to glucosuria. This is the first pharmacological class, which lowers glucose independent of the actions of insulin. The loss of glucose in the urine also leads to a negative caloric balance and a reduction in total body mass including epicardial fat. A myriad of studies have investigated the potential mechanisms underlying these effects (Fig. 3). These include effects related to the anti-hyperglycemic action but also glucose-independent effects such as a reduction in ROS, inflammation and fibrosis, altogether leading to renal and cardiac protection (Fig. 3) (Filippatos et al. 2019). However, there are some points to consider. The glucose lowering effect of SGLT-2 inhibitors occurs only down to an eGFR of <40 mL/min, but the blood pressure lowering effect is still evident if eGFR is <25 mL/min. The hemodynamic

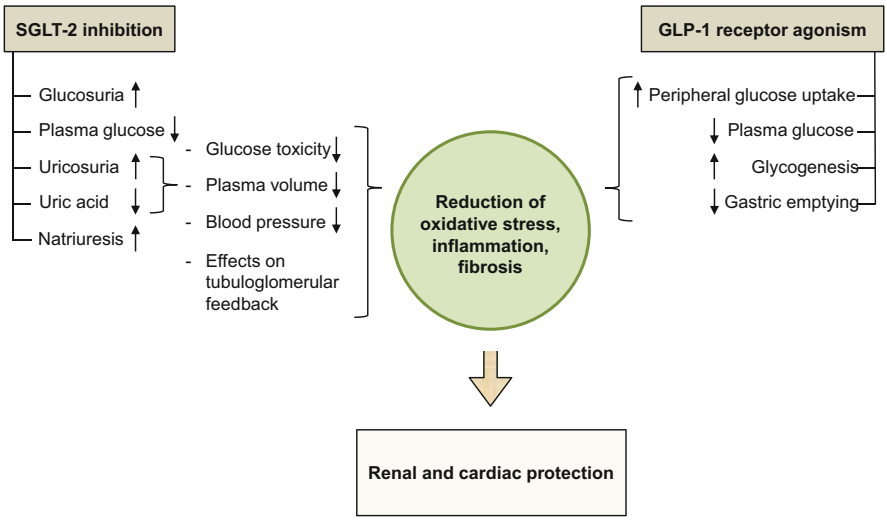

Fig. 3 SGLT-2 inhibition and GLP-1 receptor agonism reduce oxidative stress, inflammation, and fibrosis. Sodium-glucose-linked transporter-2 (SGLT-2) inhibitors increase glucosuria, uricosuria, and natriuresis, and decrease plasma glucose and uric acid levels. Furthermore, SGLT-2 inhibitors decrease glucose toxicity, plasma volume, and blood pressure, and alter the tubuloglomerular feedback. Agonism of glucagon-like peptide-1 (GLP-1) receptor increases peripheral glucose uptake and glycogenesis, while decreasing plasma glucose and gastric emptying. SGLT-2 inhibition and GLP-1 receptor agonism both result in the reduction of oxidative stress, inflammation, and fibrosis

effects often lead to an initial reduction in eGFR in the first week, which then stabilizes toward baseline measurements and provides long-term renoprotection. The cardiac protection may also occur due to inhibitory effects on sympathetic nerve activity (Gueguen et al. 2020). It has been postulated that SGLT-2 inhibitors modulate cardiac metabolism (glucose versus fatty acid consumption), which explains part of the beneficial cardiac effects. Furthermore, either neutral or favorable effects on plasma lipids have been observed. The most recent meta-analysis by McGuire et al. included six trials with this new therapeutic class and despite a degree of heterogeneity across the various compounds concerning cardiovascular outcomes, the authors found consistent protection against renal endpoints and hospitalization for heart failure (McGuire et al. 2021). It should be noted that most of these trials included a relatively healthy renal population with an eGFR of 60–90 mL/min, moderately increased albuminuria and only a very low number of patients with significantly reduced renal function. More recently, two trials have examined the renal outcomes in patients with a significantly reduced eGFR at baseline (CKD3) with macroalbuminuria. The CREDENCE study using canagliflozin included the primary endpoint of ESRD, sustained eGFR <15 mL/min/1.73 m^2 and doubling of serum creatinine or death (Perkovic et al. 2019). The study was terminated early due to clear benefits, with a 30% reduction of the primary endpoint, 32% lower relative risk for ESRD, and significantly lower heart failure and cardiovascular death. There

was no increase in amputations or fracture risk as previously reported with canagliflozin. The other landmark study, DAPA-CKD, investigated dapagliflozin (Perkovic et al. 2019). Again, the SGLT-2 inhibitor reduced the risk of sustained reduction in eGFR with reduced progression to ESRD or death from renal or cardiovascular causes and led to a 29% reduction in risk of death from heart failure and cardiovascular causes irrespective of diabetes. Dapagliflozin is also the only SGLT-2 inhibitor, which reduced all-cause mortality (31% relative risk reduction) (McMurray et al. 2021). There is now increasing evidence that the renoprotective and cardioprotective effects of SGLT-2 inhibitors also may occur in the nondiabetic context, with ongoing studies investigating this issue. In summary, a total of 5 trials demonstrated unequivocal benefits of SGLT-2 inhibitors in primary and secondary kidney disease prevention in diabetes, even in patients with low eGFR. This is now included in the *ADA Standards in Medical Care 2021*, which supports the use of SGLT-2 inhibitors in CKD or heart failure irrespective of glucose control or metformin use (American Diabetes Association 2021).

GLP-1 Receptor Agonists

Another new class of anti-diabetic agents are the glucagon-like peptide (GLP-1) receptor agonists. The mechanism of action includes increased peripheral glucose uptake as well as glycogen synthesis delaying gastric emptying and promoting satiety. This type of drug also confers multiple beneficial renal, cardiac, and metabolic effects (Fig. 3). In particular, due to the effects on gastric emptying and satiety, weight loss is observed. Furthermore, there are reductions in blood pressure and improved lipid profiles. More recent clinical trials have shown attenuation of CKD progression and reduction in cardiovascular mortality. The analysis of renal outcomes of cardiovascular outcome trials has recently shown a slowing of CKD progression (Table 1) (Holman et al. 2017; Marso et al. 2016a; Gerstein et al. 2019; Schnell et al. 2020). However, no trial has investigated renal outcomes as the primary endpoint so far. Post-hoc analyses and recent meta-analysis however suggest that GLP-1 receptor agonists reduce CKD progression. The FLOW study (effect of semaglutide versus placebo on the progression of renal impairment in subjects with type 2 diabetes and CKD) is ongoing and will further investigate this effect (Novo Nordisk A/S 2021). The AWARD-7 trial investigates dulaglutide versus insulin glargine in patients with type 2 diabetes and CKD (Tuttle et al. 2018a). These patients had already a significantly reduced eGFR at baseline. Compared to insulin, dulaglutide was associated with a lower decline in eGFR in two dose groups (-0.7 and -0.5 mL/min/1.73 m^2) compared to -3.3 mL/min/1.73 m^2 eGFR decline in the insulin-treated group. Fewer patients in the high-dose dulaglutide group reached the composite endpoint of ESRD or a >40% decline in eGFR. The LEADER trial (Liraglutide Effect and Action in Diabetes: Evaluation of Cardiovascular Outcome Results) and the SUSTAIN-6 study (trial to evaluate cardiovascular and other long-term outcomes with semaglutide in subjects with type 2 diabetes) further supported the cardioprotection by GLP-1 receptor agonists (Marso et al. 2016a, b). The REWIND study was the first study to trial weekly injections with GLP-1 receptor agonists on cardiovascular outcomes (Eli Lilly and Company 2018).

All 3 trials reported significant reductions in the secondary composite renal endpoint of up to 30%. Similar results were observed in the EXSCEL study, which showed a 40% reduction in combined renal endpoints (Bethel et al. 2018). These studies suggest – although mainly in secondary outcome analyses – that GLP-1 receptor agonists confer cardio- and renoprotection. The effects of an exendin-based GLP-1 receptor agonist efpeglenatide was recently investigated on cardiovascular and renal outcomes. Patients had type 2 diabetes with a history of cardiovascular disease and a reduced eGFR with 25–59.9 mL/min/1.73 m^2. Efpeglenatide reduced major adverse cardiovascular events (MACE) by 7% versus 9.2% in the placebo group (Gerstein et al. 2021). The composite renal endpoint occurred in 13% in the efpeglenatide-treated group versus 18.4% in the placebo group (Gerstein et al. 2021). Given the renoprotective effects observed with both drug classes, SGLT-2 inhibitors and GLP-1 receptor agonists, it has been speculated that the combination of both would lead to even better outcomes. There is a potential for synergistic effects given that the mechanism of action only partially overlaps (Fig. 3). This is currently analyzed in the EMPA-SEMA trial (Steno Diabetes Center Copenhagen 2019).

Incretin Therapies

GLP-1 receptor agonists stimulate insulin secretion and suppress glucagon secretion during hyperglycemia. Glucose-dependent insulinotropic polypeptide (GIP) not only stimulates insulin secretion in hyperglycemia, but also stimulates glucagon release during hypoglycemia. It has been hypothesized that a dual agonist for both GLP-1 and GIP receptors could enhance glycemic control and minimize hypoglycemia in patients with type 2 diabetes. Such a dual agonist, tirzepatide, has recently been studied in 2 clinical trials (Rosenstock et al. 2021; Frias et al. 2021). Given subcutaneously once weekly compared to placebo, tirzepatide reduced body weight by 7–9 kg, reduced HbA1c by 2% points, and was not associated with severe hypoglycemia (Rosenstock et al. 2021). In another trial, three doses of tirzepatide were compared to the GLP-1 receptor agonist semaglutide and demonstrated better HbA1c reduction and superior weight loss with tirzepatide (Frias et al. 2021). Whether these improvements also lead to a better renal outcome needs to be analyzed in further studies.

3.2.2 Blood Pressure

Hypertension and diabetes are named the “two bad companions” and both accelerate the development and progression of diabetic nephropathy (Fig. 2). Hypertension per se is a leading cause of CKD. A reduction in systolic blood pressure by 10 mmHg resulted in a 17% risk reduction of mortality, an 11% reduction in cardiovascular events, and 17% reduction in the development of albuminuria (Sleight 2000). In patients with type 1 or type 2 diabetes who have already developed CKD, there is clear evidence that angiotensin-converting enzyme (ACE) inhibitors or angiotensin receptor blockers (ARBs) delay the worsening of kidney function and lead to reduction in albuminuria. Albuminuria is both a risk marker and a therapeutic target to reduce the risk of further progression of renal failure. Thus, a reduction in albuminuria achieved by renin-angiotensin-aldosterone system (RAAS) blockade

will ultimately result in better renoprotection (de Zeeuw et al. 2004). However, there is a small group of diabetic patients with nephropathy who progress to ESRD without albuminuria (Macisaac and Jerums 2011). The blood pressure targets in people with diabetes are <140/90 mmHg and lower blood pressure targets are recommended in patients with macroalbuminuria or overt proteinuria (<130/80 mmHg), to reduce not only the risk for renal but also cardiovascular complications. ACE inhibitors and ARBs are the recommended first-line treatment for blood pressure control in patients with hypertension and diabetes, with a reduced kidney function <60 mL/min/1.73 m^2 and macroalbuminuria <300 mg/g (American Diabetes Association 2021).

Angiotensin-Converting Enzyme (ACE) Inhibitors

The initial studies by Lewis et al. demonstrated evidence for a renoprotective effect of ACE inhibitors in type 1 diabetes beyond their blood pressure reducing action (Lewis et al. 1993). A meta-analysis of non-hypertensive patients with type 1 diabetes and microalbuminuria showed that treatment with the ACE inhibitors decreased progression to macroalbuminuria and increased the chance of regression to normoalbuminuria (The ACE Inhibitors in Diabetic Nephropathy Trialist Group 2001).

Angiotensin II Receptor Blockers

The landmark studies, the RENAAL and IDNT studies, support the renoprotective effects of irbesartan and losartan in people with type 2 diabetes with a risk reduction of 25–28% and a 35% decline in proteinuria (Brenner et al. 2001; Lewis et al. 2001). Not only was a reduction in microalbuminuria by 38% observed, but 34% of patients with microalbuminuria regressed to normoalbuminuria (Parving et al. 2001). These renoprotective effects were independent of effects on blood pressure. The MARVAL study also showed a significant benefit of ARBs in patients with significant albuminuria as they reduced the decline in renal function by 4–5 mL/min/1.73 m^2 per year (Viberti et al. 2002). Given that the normal decline in eGFR in a healthy person is 0.8 mL/min/1.73 m^2 per year, there remains a significant residual risk. The combination of an ACE inhibitor and an ARB was investigated in the ONTARGET study, but the combination did not confer additional benefits, and on the contrary was associated with a faster decline in renal function and hyperkalemia (Mann et al. 2008).

Mineralocorticoid Receptor Antagonists

The mineralocorticoid receptor (MR) is the downstream receptor of the renin-angiotensin system (RAS) and is activated by aldosterone. Aldosterone has deleterious effects on sodium retention, blood pressure as well as cardiac and renal inflammation and fibrosis. In patients on long-term RAS blockade, there is evidence for increased aldosterone plasma levels, which is also called "aldosterone escape," resulting in ongoing inflammation and fibrosis despite long-term RAS blockade. In experimental settings, MR antagonism exerts anti-inflammatory and anti-fibrotic effects on the kidney, heart, and vasculature. In patients, MR antagonists have been shown to confer beneficial effects on heart failure but not many studies have

been performed in CKD. Furthermore, spironolactone and to a lesser degree eplerenone have been associated with side effects such as hyperkalemia and gynecomastia. A third generation MR antagonist, finerenone, has stronger affinity and potency compared to spironolactone and eplerenone. The largest study, FIDELIO-CKD is a phase 3 double-blinded randomized study in type 2 diabetic patients with moderate to severe CKD who were on maximally tolerated RAS blockade (Bakris et al. 2020). Finerenone was associated with an 18% relative risk reduction in the primary renal outcome and a 14% relative risk reduction outcome in the secondary cardiac outcome. The beneficial effects on cardiac outcomes were already seen in the first month, whereas the benefits on renal outcomes did not emerge until 12 months of treatment. Despite these advances in treatment options, a large proportion of patients still progresses to ESRD and the target of achieving a near-normal decline in renal function in diabetes has not been achieved. Thus, there remains a significant residual risk and unmet medical need. In contrast, targeting renin directly with aliskiren has not led to superior renoprotection (Parving et al. 2012). However, the endothelin receptor inhibitor avosentan showed promise in experimental animal studies but did not further reduce DKD progression in clinical trials (Mann et al. 2010). Furthermore, these non-specific endothelin receptor blockers were associated with significant side effects such as edema formation or heart failure (Mann et al. 2010). Newer, more selective endothelin receptor antagonists such as atrasentan still hold promise in selected subsets of patients who are not at risk for heart failure and fluid retention. In the recent SONAR study, patients with type 2 diabetes and albuminuria who responded well to initial dosing with a >30% in albuminuria and no substantial fluid retention demonstrated large reductions in albuminuria and reduced the risk of renal events. More recently, a post hoc analysis of the SONAR trial compared 6 weeks of SGLT-2 inhibitor combined with atrasentan versus atrasentan alone. The combination was superior on body weight reduction, fluid retention, and reduced albuminuria further (Heerspink et al. 2021).

3.3 Lipid Management

Elevated cholesterol and triglycerides are part of the diabetic milieu. Current guidelines suggest that patients with CKD, already at increased risk for cardiovascular mortality, should be treated to a low-density lipoprotein (LDL) level below 2.5 mmol/L (Colhoun et al. 2004). In a recent meta-analysis, statins have been shown to reduce albuminuria, specifically in type 2 diabetic nephropathy but the effect on long-term renal function was less clear (Shen et al. 2016). In addition, fibrates have been shown to lower albuminuria in diabetic nephropathy (Davis et al. 2011). A mild increase in serum creatinine has been observed with fibrates, which is not associated with worse renal outcome but related to creatinine excretion. Recently, monoclonal antibodies against proprotein convertase subtilisin kexin 9 (PCSK9) have been developed and these drugs lower LDL by >60%. The LDL lowering capacity is independent of baseline kidney function (Schmit et al. 2019). The effect of PCSK-9 inhibitors on kidney disease and in particular diabetic nephropathy needs to be evaluated in further studies.

4 Novel Experimental Drug Targets for DKD

Multiple signaling pathways are critically involved in the development and the progression of DKD and are potentially suitable to act as novel drug targets for DKD. Here, we provide an overview of studies that particularly aim to target signaling pathways involved in carbonyl and oxidative stress as well as inflammation. Several clinical trials using agents with anti-fibrotic properties have also been performed and many are still ongoing, however, any anti-fibrotic therapy needs to be assessed with respect to potential side effects on the immune system and on wound healing.

4.1 Carbonyl and Oxidative Stress

4.1.1 AGE-RAGE

The diabetic milieu is associated with increased formation of AGEs including early reactive and highly toxic intermediates such as methylglyoxal (MG). AGEs lead to cross-linking of proteins and DNA, thus altering their structure and function (Fig. 4).

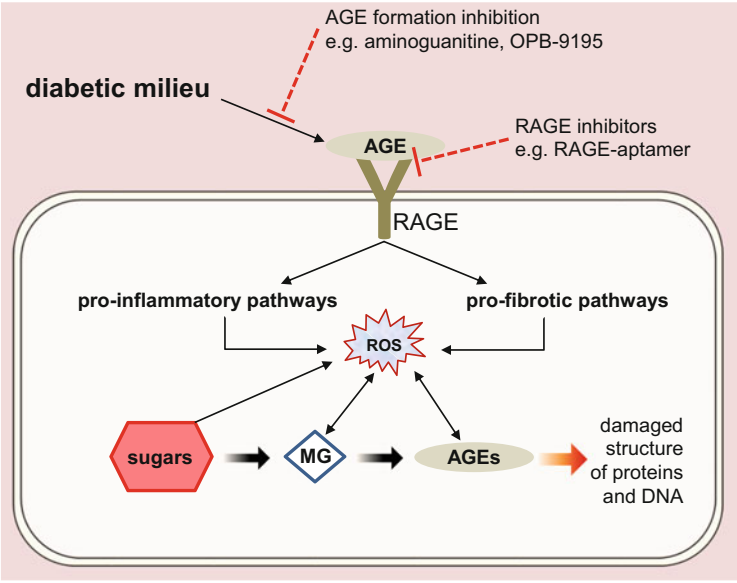

Fig. 4 Activation of the AGE-RAGE axis leads to oxidative stress, inflammation, fibrosis, and apoptosis. Increased advanced glycation end product (AGE) levels in the diabetic milieu bind to the receptor for AGEs (RAGE), resulting in the activation of pro-inflammatory and pro-fibrotic pathways, thereby elevating reactive oxygen species (ROS). Upon cytosolic hyperglycemia, sugars can be transformed to carbonyl intermediates, such as methylglyoxal (MG), and can be further modulated leading to AGEs that bind and cross-link to proteins and DNA, damaging the structure and function of proteins and DNA. The inhibition of AGE formation in the diabetic milieu as well as the blockade of RAGE are two therapeutic approaches aiming to prevent the harmful effects of the AGE–RAGE axis activation on the kidney

Furthermore, AGEs interact with their receptor, receptor for AGE (RAGE), which activates pro-inflammatory and pro-fibrotic signaling pathways. The AGE-RAGE axis plays a critical role in the pathogenesis of diabetic nephropathy as several preclinical studies have demonstrated that its activation induces oxidative stress, inflammation, and apoptosis associated with kidney function impairment (reviewed in Sanajou et al. 2018). Based on these facts, extensive research has been performed to find ways of blocking the AGE-RAGE axis, and two main approaches have evolved, the inhibition of AGE formation and the inhibition of RAGE.

AGE Formation Inhibitors

Several preclinical studies with AGE formation inhibitors, including aminoguanidine, OPB-9195, ALT-946, LR-90, salvianolic acid A or fluorofenidone (AKF-PD), have shown renoprotective effects in experimental animal models of diabetes such as streptozotocin (STZ)-induced diabetic rats, OLETF rats, or Zucker diabetic fatty rats (Nakamura et al. 1997; Wilkinson-Berka et al. 2002; Figarola et al. 2008; Qin et al. 2019; Hou et al. 2017). In all studies, a reduction in albuminuria was the most prominent effect. Aminoguanidine, OPB-9195, LR-90, salvianolic acid A, and AKF-PD furthermore led to reduced formation of glomerulosclerosis, while ALT-946 and LR-90 also demonstrated prevention of tubular fibrosis and damage (Nakamura et al. 1997; Wilkinson-Berka et al. 2002; Figarola et al. 2008; Qin et al. 2019; Hou et al. 2017). Furthermore, vitamins like pyridoxamine (derivative of vitamin B6), thiamine (vitamin B1), and benfotiamine (prodrug of vitamin B1) are known to prevent AGE formation and were also shown to lead to attenuated albuminuria when given to STZ-induced diabetic rats (Degenhardt et al. 2002; Babaei-Jadidi et al. 2003). Thiamine and benfotiamine treatments additionally reduced oxidative stress and inflammatory signaling pathways, such as the activation of protein kinase C (PKC), and benfotiamine alone also inhibited diabetes-induced glomerular hyperfiltration (Babaei-Jadidi et al. 2003). In a pilot trial with type 2 diabetes patients (PYR-210), pyridoxamine treatment showed a trend towards reduced creatinine levels, which is in line with observed decreased serum creatinine levels in diabetic rats treated with pyridoxamine (Degenhardt et al. 2002; Dwyer et al. 2015). Whether pyridoxamine has also positive effects on primary renal end points like albuminuria in humans as it does in animal models needs to be learned from larger future prospective studies with diabetic patients (such as PIONEER-CSG-17). Another approach was conducted in a study using alagebrium, which is an AGE inhibitor and putative AGE-protein crosslink breaker. Its administration to STZ-injected diabetic *ApoE* knockout mice showed similar effects in the kidney like in other studies with AGE formation inhibitors as it reduced albuminuria and glomerulosclerosis formation (Watson et al. 2012). This study moreover highlighted that alagebrium acts also RAGE-independent and that important RAGE-independent signaling pathways are activated by AGEs in the kidney and contribute to DKD, as treatment of *Rage/ApoE* double knockout mice with alagebrium still attenuated glomerulosclerosis, inflammation, and oxidative stress in the renal cortex (Watson et al. 2012).

RAGE Inhibitors

In contrast to the AGE formation inhibitors, few studies have been performed with RAGE inhibitors that prevent binding of AGEs to their receptor (Fig. 4). In an early study, an antibody against RAGE has been applied to STZ-induced diabetic mice, resulting in reduced albumin excretion and improved creatinine clearance when compared to control-treated diabetic mice (Jensen et al. 2006). Other RAGE inhibitors such as RAGE-aptamer or FPS-ZM1 showed similar effects in STZ-induced diabetic rats or AGE-loaded diabetic mice, respectively, and were characterized by reduced albuminuria as well as less inflammation and fibrosis in the kidney (Matsui et al. 2017). The RAGE-aptamer furthermore had preventive effects on oxidative stress generation because NADPH oxidase (NOX) activity was reduced in diabetic rats treated with this RAGE inhibitor (Matsui et al. 2017). More recently, it has been shown that transactivation of RAGE mediates the pro-inflammatory signaling of angiotensin II and mutant RAGE ligands have been able to attenuate this transactivation, opening new avenues for RAGE inhibition (Pickering et al. 2019). Overall, the majority of all conducted preclinical studies have provided evidence for the renoprotective effects of inhibitors that target the AGE-RAGE axis. Clinical trials including diabetic patients will need to be performed to investigate whether inhibitors of the AGE-RAGE axis have similar beneficial effects in humans and could be used as a new therapeutic approach for DKD.

4.1.2 NOX

The NOX family consists of seven isoforms, including NOX1 to NOX5 as well as dual oxidases 1 and 2 (DUOX1 and DUOX2). NOXs are transmembrane proteins and share the ability to transfer an electron across a membrane to reduce oxygen to superoxide or hydrogen peroxide. The biological meaning of NOX-derived ROS compromises microbial defense mechanisms, posttranslational protein processing, cellular signaling, regulation of gene expression, and cell differentiation (Bedard and Krause 2007). NOX activity is predominantly regulated on transcriptional and translational level and has very low or no constitutive activity. Under pathological conditions however, including hypertension and diabetes, enzyme activation can increase and overcome the antioxidative capacity of the cell, causing oxidative stress and hence tissue damage. The human kidney expresses NOX1, NOX2, NOX4, and NOX5, which are found in glomerular, endothelial and mesangial cells, podocytes, proximal and distal tubular cells as well as in interstitial fibroblasts (Jha et al. 2016a; Gorin et al. 2005). Yet, not all NOXs contribute equally to DKD. Studies with deletion of the *Nox1* gene in STZ-induced diabetic *ApoE* knockout mice have not led to improved albuminuria or reduced mesangial expansion, however, were associated with reduced atherosclerosis (Jha et al. 2014). Similarly, *Nox2* deletion in a diabetic mouse model has revealed comparable albuminuria and mesangial expansion levels between diabetic and nondiabetic mice (You et al. 2013), suggesting that the isoforms NOX1 and NOX2 do not critically contribute to DKD. In contrast, several preclinical studies have demonstrated that particularly NOX4 and NOX5 are involved in the development and progression of DKD (Jha et al. 2017).

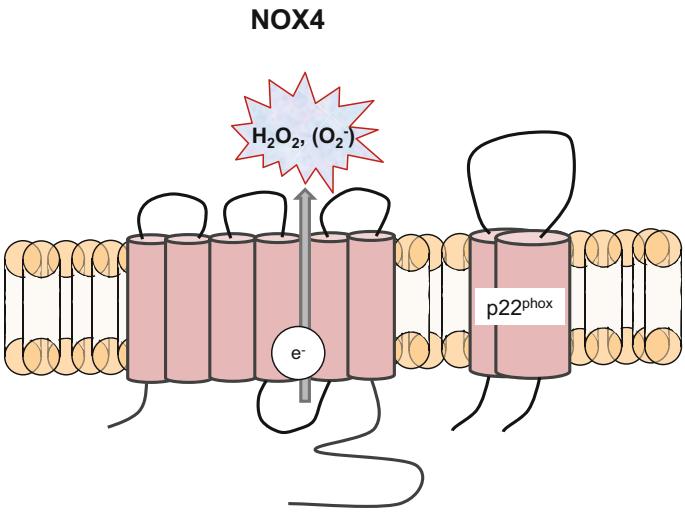

Fig. 5 Simplified model of the NADPH oxidase 4 (NOX4) complex. NOX4 localizes in membranes with six transmembrane domains and requires $p22^{phox}$ for activation. The complex transfers an electron from NADPH across the membrane to produce ROS, such as hydrogen peroxide (H_2O_2) and to a lesser extent superoxide (O_2^-)

NOX4 as a New Target in DKD

Due to its abundance in renal tissue, NOX4 was originally termed Renox (renal oxidase) (Fig. 5) (Geiszt et al. 2000). NOX4 can be found in glomerular endothelial cells, mesangial cells, podocytes, and proximal tubular epithelial cells of the kidney, where it predominantly produces hydrogen peroxide (Fig. 5) (Jha et al. 2016a; Rajaram et al. 2019; Martyn et al. 2006). Different to NOX1 or NOX2 that are located in the plasma membrane and produce extracellular superoxide, NOX4 mainly localizes in intracellular membranes and compartments, such as endoplasmic reticulum, nucleus, mitochondria, and the cytoskeleton, which might explain the challenge of measuring superoxide, hypothesizing that the products of NOX4 are converted to hydrogen peroxide, being able to pass membranes and becoming measurable (Bedard and Krause 2007; Block et al. 2009; Chen et al. 2008; Takac et al. 2011). It has been shown that the E-loop of NOX4 is 28 amino acids longer than that of other NOXs, and minor alterations in that loop can switch superoxide production mode to hydrogen peroxide production mode (Takac et al. 2011). Several studies show upregulation of *Nox4* expression and increased ROS levels in podocytes, mesangial cells, and proximal epithelial tubular cells upon high-glucose conditions (Gorin et al. 2005; Jha et al. 2014; Sedeek et al. 2010). Global *Nox4*-deleted STZ-induced diabetic *ApoE* knockout mice exhibited renoprotective effects, such as reduced albuminuria, attenuated glomerular macrophage infiltration as well as decreased levels of monocyte chemoattractant protein-1 (MCP-1) and nuclear factor-kappa B (NF-κB) (Jha et al. 2014). Consequently, in diabetic mice, NOX4 has been identified as the main source of ROS (Jha et al. 2014). Further studies engaged

in the role of NOX4 in STZ-induced diabetic rats with 2 weeks of treatment with phosphorothioated antisense (AS) nucleotides against *Nox4 (*Gorin et al. 2005*)*. The findings have shown not only downregulated NOX4 levels in the renal cortex in AS-treated diabetic rats but also a reduction of fibronectin as well as whole kidney and glomerular hypertrophy (Gorin et al. 2005). In vitro studies with mesangial cells also showed reduced levels of NOX4 and fibronectin upon AS treatment (Gorin et al. 2005). The beneficial effects of global NOX4 absence have similarly been replicated in podocyte-specific *Nox4*-deleted STZ-induced diabetic mice (Jha et al. 2016b). The absence of NOX4-derived ROS in podocytes led to renoprotective effects including reduction of fibrotic markers such as collagen IV and fibronectin as well as inflammatory markers such as MCP-1 and PKC-α. Additionally, a reduction of mesangial expansion, glomerulosclerosis, GBM thickness, albuminuria, expression of VEGF-A, and restoration of nephrin levels could be measured, which emphasizes the important contribution of NOX4 activity to DKD pathogenesis (Jha et al. 2016b). Interestingly, NOX4 deletion from proximal tubules did not confer renoprotection in diabetes despite significant alterations in mitochondrial ROS formation (Thallas-Bonke et al. 2021), suggesting different pathogenic mechanisms for proximal tubular changes as opposed to glomerular changes.

NOX5 as a New Target in Human DKD

Another important NOX isoform in the context of DKD is NOX5 (Fig. 6). Although NOX5 shares significant homology to NOX1 and NOX2, NOX5 is structurally distinct and expressed in five splice variants (NOX5α-δ and NOX5S) with NOX5α and NOX5β responsible for superoxide production (Fulton 2009; Serrander et al. 2007). The human NOX5 isoform is not endogenously expressed in rodents, and therefore its experimental investigation has been challenging. STZ-induced diabetic transgenic mice expressing human *Nox5* in renal mesangial or endothelial

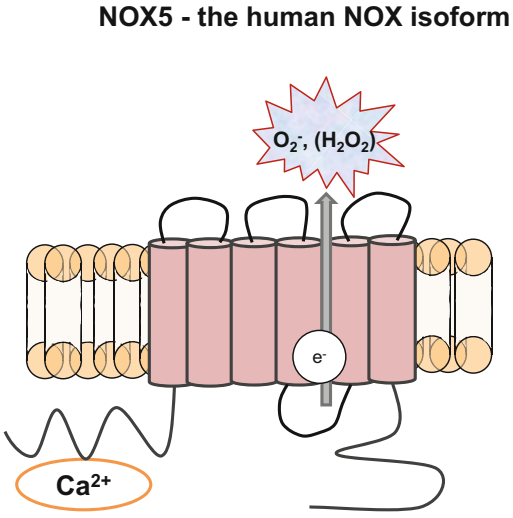

Fig. 6 Simplified model of the NADPH oxidase 5 (NOX5) complex. NOX5 localizes in membranes with six transmembrane domains. Calcium ions (Ca^{2+}) are required to activate NOX5 by binding to the N-terminal side. The complex transfers an electron from NADPH across the membrane to produce ROS, such as superoxide (O_2^-) and potentially hydrogen peroxide (H_2O_2)

cells have shown to accelerate glomerulosclerosis, mesangial expansion, and ECM accumulation by collagen IV and fibronectin overproduction (Jha et al. 2017). In accordance with human kidney biopsies, where NOX5 was found to be increased in patients with diabetes, silencing of *NOX5* in in vitro studies using human mesangial cells showed a reduction of high-glucose- and TGF-β-induced ROS (Jha et al. 2017; Holterman et al. 2014). Furthermore, podocyte-specific *Nox5* expression in transgenic mice led to an early onset of albuminuria even under nondiabetic conditions and resulted in even worse effects after diabetes was induced by STZ injections (Holterman et al. 2014). Further studies using animal models expressing NOX5 such as the rabbit, which expresses all NOX isoforms including NOX5 as in humans, are required (Serrander et al. 2007).

NOX Inhibitors

Based on the relevance of NOX in DKD, different NOX inhibitors have been developed in the past to be used as potential therapeutics in DKD (reviewed in Urner et al. 2020), and more specific inhibitors that target certain NOX isoforms, such as NOX5, are being currently developed. In line with preclinical genetic studies using knockout mouse models, the pharmacological inhibition of NOX by inhibitors has generally confirmed renoprotective effects when used in diabetic mice or rats. The NOX1/NOX4 inhibitor GKT137831 has already been widely tested in different diabetic mouse models. In STZ-injected diabetic *ApoE* knockout mice, treatment with GKT137831 led to attenuation of albuminuria, glomerulosclerosis as well as inflammation and oxidative stress in the kidney (Jha et al. 2014; Gray et al. 2017). In line with this, complementary in vitro experiments using human podocytes showed that GKT137831 treatment reduces ROS generation and the expression of fibrotic markers upon diabetic conditions (Jha et al. 2014). Importantly, the renoprotective effects of GKT137831 are very likely due to its inhibition of certainly NOX4, as genetic studies showed that deletion of *Nox4*, but not *Nox1*, leads to significant improvements of functional and structural renal characteristics in diabetic mice (Jha et al. 2014). Similar effects of GKT137831 were observed in OVE26 and Akita mice (Gorin et al. 2015; You et al. 2016), supporting its renoprotective properties in type 1 diabetes models. In line with this, another NOX1/NOX4 inhibitor, GKT136901, efficiently reduced albuminuria, glomerulosclerosis, and tubular damage when given to *db/db* mice (Sedeek et al. 2013), demonstrating that inhibition of NOX4 might be a promising target in both type 1 and type 2 diabetes. Based on the promising preclinical data, a short-term phase II clinical trial has been performed in which type 2 diabetes patients with DKD have been treated with the inhibitor GKT137831 for 12 weeks (NCT02010242). However, the inhibitor failed to meet the primary endpoint of reducing albuminuria, although several secondary efficacy endpoints were met. One reason why the inhibitor was not as efficient in patients could be that in this study only patients with very advanced kidney disease who also were on maximum blockade of RAAS were treated with GKT137831 for a short time of 12 weeks. In addition, particularly GKT137831 showed most promising effects in preclinical studies when applied to type 1 diabetes models, while DKD in type 2 diabetes patients is much more heterogenous. Another long-term phase II clinical

trial is currently running with type 1 diabetes patients who have a persistent albuminuria and a preserved renal function (ACTRN12617001187336). These patients are receiving GKT137831 treatment for a total of 48 weeks on top of stable RAAS blockade in higher doses. The primary endpoint of the study is the change in the albumin-creatinine-ratio, while the secondary endpoints include the eGFR and changes in inflammatory and fibrotic markers. This study will reveal whether inhibition of NOX1/NOX4 by GKT137831 has also beneficial effects on DKD in type 1 diabetes patients (Reutens et al. 2020). Other NOX inhibitors that are available are not specific to a certain NOX isoform. For example, apocynin downregulates intracellular ROS levels and inhibits the downstream signaling of all NOX isoforms (Altenhofer et al. 2015). However, it should be noted that its beneficial therapeutic effects in preclinical studies may not only be due to direct NOX inhibition (Heumuller et al. 2008). In STZ-induced diabetic Sprague-Dawley rats, treatment of apocynin attenuated diabetes-induced albuminuria and glomerulosclerosis (Thallas-Bonke et al. 2008). It particularly reduced the expression of fibronectin and collagen IV, and furthermore led to reduced PKC-α signaling, underlining anti-fibrotic as well as anti-inflammatory properties of apocynin in the diabetic kidney (Thallas-Bonke et al. 2008). Similar observations have been made in other studies with STZ-induced diabetic rats, which received apocynin and showed reduced ECM protein expression and thus less mesangial expansion, glomerulosclerosis, and interstitial fibrosis (Asaba et al. 2005; Xin et al. 2018). The novel pan-NOX inhibitor APX-115 also blocks all NOX isoforms, and several studies indicate that it has beneficial effects when used to treat DKD in preclinical animal models. Treatment of STZ-induced diabetic mice led to reduction in urinary albumin excretion, creatinine clearance, glomerular hypertrophy, glomerulosclerosis, tubular injury, podocyte injury as well as inflammation and oxidative stress, and furthermore improved mitochondrial and peroxisomal function in the kidney (Kwon et al. 2017). In *db/db* mice, similar observations have been made in terms of decreased albuminuria and preserved creatinine levels as well as reduced oxidative stress in diabetic mice treated with APX-115 in comparison with control-treated diabetic mice (Cha et al. 2017). In a recent study, it has been furthermore shown that APX-115 has renoprotective effects also through NOX5 inhibition. As NOX5 is not endogenously expressed in rodents, *Nox5* transgenic (podocyte-specific) high-fat diet fed diabetic mice were treated with APX-115, which blocked the diabetes-induced upregulation of NOX5 expression and led to reduced urinary albumin-creatinine-levels, renal fibrotic events, and inflammation (Lee et al. 2020). In summary, different inhibitors for NOX signaling have been widely studied in the context of DKD in animal models and turned out to have beneficial effects. Follow-up clinical trials with those inhibitors would reveal whether similar results can be observed in diabetes patients. Moreover, given the proven evidence from preclinical studies on the critical contribution of NOX5 to the development and progression of DKD, more specific inhibitors that target NOX5 should be developed and tested for a potential therapeutic use in DKD patients.

4.1.3 Xanthine Oxidoreductase

Xanthine oxidoreductases (XORs) have multiple activities, including xanthine dehydrogenase, xanthine oxidase, NADH oxidase, or nitrite reductase activity, responsible for uric acid formation as well as ROS and NO generation (reviewed in Bortolotti et al. 2021). Increased serum uric acid levels, driven by increased XOR activity, are considered as a risk factor for the development as well as progression of DKD in both type 1 and type 2 diabetes patients (Hovind et al. 2009; Zoppini et al. 2012). Furthermore, XOR activity likely is associated with ESRD, as particularly patients with CKD who require dialysis show upregulated circulating XORs (Boban et al. 2014). Several preclinical and clinical studies have investigated the effects of XOR inhibitors on DKD. Allopurinol is a specific inhibitor of xanthine oxidase and decreases serum uric acid formation (reviewed in Pacher et al. 2006). Treatment of diabetic db/db mice with allopurinol resulted in decreased uric acid levels, reduced albuminuria as well as ameliorated tubulointerstitial injury, but did not lead to changes in mesangial expansion or glomerulosclerosis (Kosugi et al. 2009). The observed beneficial renal effects are likely due to the reduction of serum uric acid by allopurinol instead of an inhibition of ROS generation by xanthine oxidase since the treatment did not reduce oxidative stress in the diabetic kidney (Kosugi et al. 2009). In two independent studies performed in diabetic Zucker obese rats or in STZ-induced diabetic Sprague-Dawley rats, treatment with another non-purine inhibitor of xanthine oxidase, febuxostat, also resulted in reduced albuminuria, respectively (Komers et al. 2016; Lee et al. 2014). Notably, both studies observed that febuxostat treatment also decreased the expression of the oxidative stress marker nitrotyrosine in the kidney of diabetic rats, suggesting that its mechanism of action in the kidney may differ from allopurinol (Komers et al. 2016; Lee et al. 2014). In patients with CKD, treatment with allopurinol reduced serum uric acid levels and delayed the progression of renal disease as shown by two small early clinical trials (Siu et al. 2006; Goicoechea et al. 2010). However, recently published high-profile long-term clinical trials in patients with CKD and a high risk of progression as in the CKD-FIX trial, or in type 1 diabetes patients with early-to-moderate DKD as in the PERL trial, did not show any benefits of serum uric acid reduction by allopurinol on renal outcomes (Doria et al. 2020; Badve et al. 2020). When patients with stage 3 and 4 CKD were treated with febuxostat for a short period of 6 months, their kidney function improved as defined by a delay of the eGFR decline (Sircar et al. 2015). However, when patients with stage 3 CKD underwent a long-term treatment with febuxostat for 18 months, no beneficial effects on the kidney function could be observed (Kimura et al. 2018). Furthermore, in patients with type 2 diabetes and DKD, febuxostat treatment for 6 months could not improve albuminuria or eGFR (Beddhu et al. 2016). However, the majority of studies suggest that XOR inhibitors represent a useful tool to reduce disease progression in CKD (Pisano et al. 2017).

4.1.4 Mitochondrial ROS

The kidney is one of the most metabolically active organs, and therefore also contains a high mitochondria content. Although mitochondria are well known as the powerhouses of the cell, they also provide an important site for ROS production

in the form of superoxide. During oxidative phosphorylation, NADH and $FADH_2$ donate their electrons into the mitochondrial inner membrane. The embedded protein complexes NADH ubiquinone oxidoreductase (complex I) and succinate dehydrogenase (complex II) accept the electrons and transfer them to ubiquinol. Coenzyme Q:cytochrome c reductase (complex III) receives the electrons from ubiquinol and uses them to reduce the electron carrier cytochrome c. The electrons are unloaded at cytochrome c oxidase (complex IV), the site where molecular oxygen is reduced to water. During this process, molecular oxygen can also be reduced with a single electron and produce superoxide anion radicals, which occurs predominantly at complex I, II, and III (Nolfi-Donegan et al. 2020). The superoxide molecules generated by the electron transport chain (ETC) are released to both the matrix and the intermembrane space (Galvan et al. 2017a). However, the ETC is not the exclusive mitochondrial source of superoxide as the mitochondrial matrix also accommodates other superoxide-producing enzymes, such as 2-oxoglutarate dehydrogenase, pyruvate dehydrogenase, and glycerol 3-phosphate dehydrogenase (Murphy 2009; Brand 2010; Coughlan and Sharma 2016). Either by spontaneous dismutation or by enzymatic activity of superoxide dismutases (SODs), superoxide is converted into hydrogen peroxide, which in contrast to superoxide is membrane-permeable. Hyperglycemia susceptible cells can experience excessive glucose influx, activating not only certain pathways in order to deal with the increased amounts of glucose, but also saturating glycolysis with ensued citric acid cycle, which produces large amounts of NADH and $FADH_2$ that feed the ETC and accelerate the superoxide leakage (Coughlan and Sharma 2016). The view of elevated glucose levels consequently leading to the overproduction of superoxide by mitochondria is still controversial and has been reviewed in Coughlan and Sharma (2016). Indeed, elevated superoxide levels have been reported in vitro in renal cells exposed to high glucose, as well as in vivo in diabetic kidney studies (Coughlan and Sharma 2016). Additionally, studies using diabetic *db/db* mice demonstrated increased mitochondrial matrix ROS (Galvan et al. 2017b). In contrast, another study involving STZ-induced diabetic mice reported a reduction of superoxide in the diabetic kidney (Dugan et al. 2013). Notably, experimental measurement of superoxide is a challenge due to its short half-life of seconds before dismutating to hydrogen peroxide (Dugan et al. 2013). The question arises whether the controversy is due to in fact low mitochondrial superoxide production or due to inadequate measurement methods. Furthermore, mitochondrial and cytoplasmic ROS may be formed in a time-dependent manner. Therefore, further studies are needed to define the dynamic changes of mitochondrial ROS in DKD. Nevertheless, there is solid evidence for the critical involvement of mitochondrial (dys)function in DKD (reviewed in Mise et al. 2020). A dysregulation of complex I, III, or IV has been previously detected in the diabetic kidney of both diabetic animal models and diabetic patients (reviewed in Mise et al. 2020). In this context, also changes in ATP production have been described for different stages of DKD (reviewed in Mise et al. 2020). Indeed, in a study with STZ-induced diabetic rats, changes in mitochondrial function and ATP synthesis appeared even before first renal tissue changes could be observed (Coughlan et al. 2016), suggesting mitochondrial dysfunction as an early

indicator for DKD. Therefore, restoring or improving mitochondrial function as well as balancing mitochondrial ROS production would likely be beneficial for slowing the pathological disease progression in the kidney. For example, the mitochondria-targeted peptide SS-31 localizes in the inner mitochondrial membrane, interacts with and stabilizes the mitochondrial phospholipid cardiolipin, and furthermore leads to reduced mitochondrial ROS levels (Mise et al. 2020). SS-31 has been shown to reduce proteinuria, glomerular hypertrophy as well as the expression of oxidative stress and fibrotic markers in the kidney of STZ-induced diabetic CD-1 mice (Hou et al. 2016), suggesting that improving mitochondrial function has renoprotective effects. Activation of the protein Sirtuin-1 (SIRT1), which is involved in regulating mitochondrial biogenesis and energetic homeostasis, by a newly developed selective SIRT1 agonist resulted also in marked reduction in albuminuria as well as glomerular injury associated with reduced podocyte loss and glomerular oxidative stress (Hong et al. 2018). Similar effects have been observed with coenzyme Q10 that plays an important role as an electron carrier during mitochondrial respiration as well as a mitochondrial endogenous ROS scavenger. Treatment of diabetic *db/db* mice with ubiquinone, the oxidized form of coenzyme Q10, resulted in reduced albuminuria and tubulointerstitial fibrosis, correlating with a normalized mitochondrial ATP synthesis and ameliorated mitochondrial hydrogen peroxide production (Sourris et al. 2012). A meta-analysis of 8 publications on clinical trials with coenzyme Q10 supplementation in patients also suggests that coenzyme Q10 can ameliorate DKD, at least when combined with other standardized therapy, such as blood pressure controlling agents (Zhang et al. 2019a). Another form of coenzyme Q10 is MitoQ that has been developed to enrich its uptake into mitochondria. In diabetic *db/db* mice, MitoQ treatment for 12 weeks also reduced albuminuria and improved the eGFR (Ward et al. 2017). Whether improvement of mitochondrial function by MitoQ acts also renoprotective in humans will hopefully be clarified by a phase 4 clinical trial with patients with CKD that is currently running (NCT02364648).

4.1.5 Antioxidants

The effects of supplemented direct antioxidants, such as vitamin A, vitamin C, vitamin E, selenium, zinc, magnesium, methionine, beta-carotene, glutathione, or ubiquinone alone or in combination, on DKD have been investigated in very heterogenous studies with different outcomes on the renal function (see (Bolignano et al. 2017)). For example, in one of the longest clinical trials of 4.5 years (HOPE), supplementation of vitamin E alone did not show any effects on the development of overt nephropathy in patients with diabetes and cardiovascular disease (Lonn et al. 2002). However, a recent meta-analysis of 15 publications revealed that antioxidant treatment significantly decreases albuminuria in patients with either type 1 or type 2 diabetes, and therefore may be preventive for early renal damage (Bolignano et al. 2017). Particularly the supplementation with vitamin C in combination with vitamin E as well as zinc or curcumin (turmeric) achieved a reduction in albuminuria in some studies including in diabetic patients (summarized in Bolignano et al. 2017).

Nrf2-KEAP

Another approach are indirect antioxidants, such as nuclear factor-2 erythroid related factor 2 (Nrf2), which is regulated by Kelch-like ECH associated protein 1 (KEAP). Nrf2 signaling activates the transcription of genes encoding antioxidant and detoxifying molecules and is an important regulator of ROS production by both NOXs and mitochondria (Kovac et al. 2015). In STZ-induced diabetic *Nrf2* knock-out mice, hyperglycemia-induced oxidative stress and renal damage is accelerated compared to diabetic wild-type controls, suggesting that Nrf2 is required for renal protection in DKD (Jiang et al. 2010). Treatment of STZ-induced diabetic mice with Nrf2-activating compounds, such as sulforaphane or cinnamic aldehyde, led to a reduction of oxidative stress and mesangial matrix expansion in renal cells and resulted in attenuated albuminuria (Zheng et al. 2011). Several other activators of Nrf2 signaling with different target mechanisms have since then been used in preclinical animal models of DKD to investigate their beneficial effects on the kidney and are summarized elsewhere (reviewed in Adelusi et al. 2020). The Nrf2 activator bardoxolone methyl has also been tested in phase 2 clinical trials with type 2 diabetes patients with CKD. While two studies with a duration of 8 weeks or 52 weeks of treatment (BEAM) showed that bardoxolone methyl improved the eGFR in diabetic patients with stage 3–4 CKD (Pergola et al. 2011a, b), a later study with type 2 diabetes patients and stage 4 CKD (BEACON) resulted in an increased rate of cardiovascular events in patients treated with bardoxolone methyl and had to be terminated after 9 months (de Zeeuw et al. 2013). Despite these safety concerns, another phase 2 clinical trial with bardoxolone methyl in type 2 diabetes patients and stage 3–4 CKD has been initiated afterwards, in which patients with a high risk for cardiovascular events have been excluded (TSUBAKI). The recently published outcome of this study revealed that treatment with bardoxolone methyl for 16 weeks resulted in significantly increased eGFR and did not lead to heart failure or death (Nangaku et al. 2020). Whether the clinical renal benefits of bardoxolone methyl treatment outweigh potential side effects in diabetic patients needs to be further evaluated in the future.

4.2 Inflammation

4.2.1 Inflammasome

The existence of a chronic low-grade renal inflammation is apparent in diabetes with its role in promoting the progression of DKD. Elements of the diabetic milieu including high-glucose and glyco/lipoxidation products stimulate immune cells and accelerate the production of pro-inflammatory cytokines, which in turn induce resident renal cells to produce a spectrum of chemokines (Fig. 7). The cytokines, particularly various interleukins such as interleukin-1β (IL-1β) and interleukin-18 (IL-18), tumor necrosis factor-α (TNF-α), and interferon-γ (INF-γ), direct the secretion of chemokines including MCP-1, intercellular adhesion molecule (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) which result into transmigration and infiltration of immune cells establishing an inflammatory cycle in the kidney

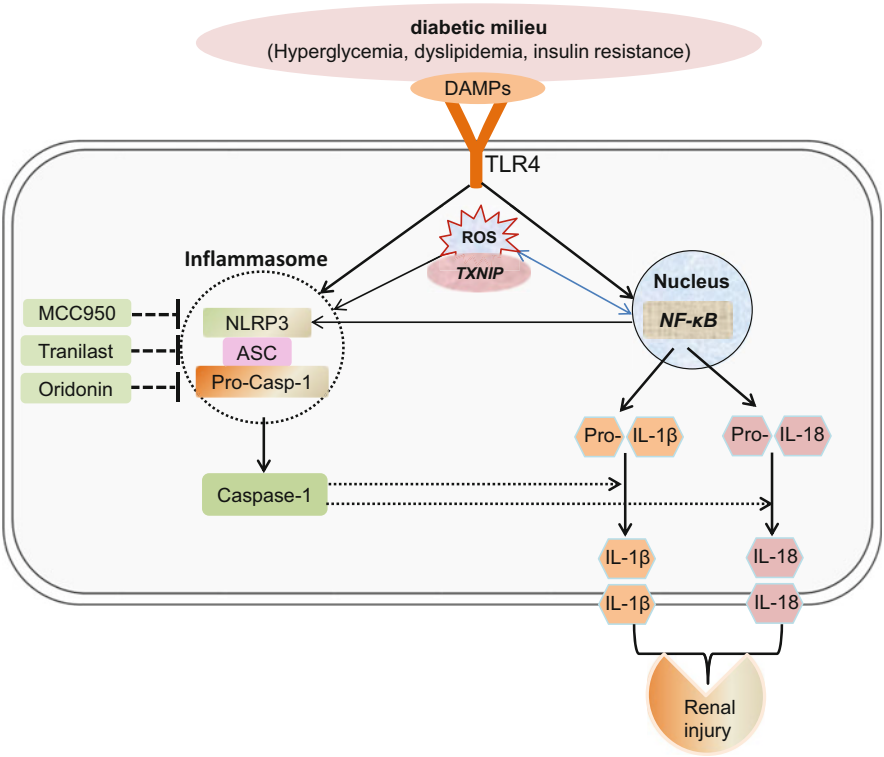

Fig. 7 Activation and inhibition of NLRP3 inflammasome in DKD. Diabetes-related DAMPs activate the NF-kB signaling pathway and the NLRP3 inflammasome via stimulation of TLR4, intracellular ROS, and the ROS-sensitive factor TXNIP. Activation of NF-kB leads to production of pro-IL-1β and pro-IL-18 cytokines. In parallel, activation of the NLRP3 inflammasome stimulates caspase-1 to form mature IL-1β and pro-IL-18 cytokines. The release of IL-1β and IL-18 cytokines creates an inflammatory environment resulting in renal cell injury in diabetes. Inhibition of NLRP3 inflammasome components (NLRP3, ASC, and pro-caspase-1) by MCC950, Tranilast, and Oridonin has been suggested to confer renoprotection in diabetes

(Lim and Tesch 2012). Moreover, the secretion of IL-1β and IL-18 in the kidney promotes the expression of adhesion molecules like ICAM-1, VCAM-1, and VEGF-A leading to systemic endothelial dysfunction, a process that promotes leukocyte adhesion and vascular leakage in the kidney (Chow et al. 2005). Among the complex network of pro-inflammatory cytokines shown to be implicated in DKD, IL-1β has been identified as a key player in initiating and promoting inflammation-induced organ dysfunction (Everett et al. 2018). A class of multi-protein complexes representing the critical components of innate immunity, known as inflammasomes, has been identified as a potential mediator in coordinating the inflammatory response in chronic diseases including DKD (Fig. 7). In response to the diabetic milieu, the assembly and thereby activation of inflammasomes direct the activation of caspase-1

leading to the secretion of the pro-inflammatory cytokines IL-1β and IL-18, subsequently resulting in pyroptosis, an inflammatory form of programmed cell death (Broz and Dixit 2016). A typical inflammasome consists of an upstream sensor protein of the NOD-like receptor (NLR) family, the adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC), and the downstream effector cysteine protease pro-caspase-1. Activation of inflammasomes by pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs, danger signal) direct the effector molecule caspase-1 to induce the conversion of pro-IL-1β and pro-IL-18 to their mature bioactive forms IL-1β and IL-18 causing the cell pyroptosis (Fig. 7) (Lamkanfi and Dixit 2014). Depending on their differences in structure and activation, several NLR proteins have been identified (NOD-, LRR-, and pyrin domain-containing 3 (NLRP3) and have been reported to play a role in regulating chronic renal inflammation and progression of DKD (Ram et al. 2020).

Activation of NLRP3 Inflammasome

Essential components of the NLRP3 inflammasome are found to be expressed in both resident renal cells (podocytes, mesangial cells, endothelial cells, and tubular epithelial cells) and immune cells, primarily macrophages and dendritic cells (Hutton et al. 2016). In contrast, the major cellular sources of IL-1β and IL-18 are the immune cells, particularly monocytes and macrophages, and certain renal cell populations, mainly tubular epithelial cells. Moreover, chronic injury to the kidney exposes renal cells to numerous inflammasome activators, such as defective autophagy, uric acid, extracellular ATP levels, and matrix degradation products shown to be implicated in DKD (Menini et al. 2020; Solini et al. 2013). In diabetic patients, higher levels of circulating and urinary IL-1β and IL-18 are linked with the NLRP3 inflammasome (Navarro-Gonzalez and Mora-Fernandez 2008). Moreover, inhibition of the NLRP3 inflammasome pathway appears to reduce inflammation and fibrosis in DKD (Yang et al. 2014). In experimental models of diabetes, activation of the NLRP3 inflammasome has been shown to promote renal injury by increased secretion of circulatory and renal IL-1β and IL-18 with enhanced albuminuria and renal fibrosis. Indeed, all these changes were significantly attenuated by genetic deletion and pharmacological inhibition of NLRP3 or caspase-1 in diabetic mice, suggesting the role for NLRP3 activation in DKD. Furthermore, not only in diabetes but also in models of acute kidney injury such as ureteral occlusion or renal ischemia/reperfusion-induced injury in mice, deficiency of NLRP3 demonstrated protection against renal tubular damage and interstitial inflammation (Vilaysane et al. 2010). In addition, activation of NLRP3 by high glucose in renal tubular cells (HK-2) was found to be associated with cleavage of caspase-1 and IL-1β leading to the release of the pro-inflammatory cytokines IL-1β and IL-18 (Garibotto et al. 2017).

TLR4-NFκB-ROS Signaling Pathway

The excessive activation of pro-inflammatory cytokines can promote the progression of renal fibrosis. It is evident from experimental studies that in DKD increased

expression of toll-like receptor 4 (TLR4) is associated with enhanced activation of NFκB and NLRP3 inflammasome leading to the release of pro-inflammatory cytokines (IL-1β and IL-18) and chemokines (MCP-1) in the kidney causing progression of DKD (Lin et al. 2012). Indeed, deficiency of *Tlr4* in mice showed attenuation of diabetes-induced increased albuminuria, renal fibrosis and interstitial macrophage infiltration via downregulation of renal NF-κB activation and MCP-1 expression (Lin et al. 2012). On the other hand, inhibition of TLR4/NF-κB signaling led to decreased expression of components of NLRP3 with less secretion of IL-1β and IL-18 in DKD, suggesting a role for the TLR4/NF-κB-inflammasome signaling pathway in DKD. The co-existence and interplay between inflammation and oxidative stress plays a critical role in creating an overwhelming inflammatory environment leading to progression of renal tissue damage and fibrosis in diabetes (Jha et al. 2018). Under diabetic conditions, enhanced renal ROS formation has been shown to modulate the activation of the NLRP3 inflammasome and subsequent kidney injury (Han et al. 2018). In addition, renal ROS in diabetes also regulates the function of the transcription factor NF-κB, which is responsible for the production of the immature pro-inflammatory cytokines pro-IL-1β and pro-IL-18, which are then cleaved by inflammasome complexes to their bioactive form causing kidney damage (Ram et al. 2020; Petrilli et al. 2007). An association between the ROS-sensitive factor thioredoxin-interacting protein (TXNIP) and NLRP3 inflammasome activation in response to high glucose has been demonstrated in renal cells with enhanced ROS production and IL-1β (Xiao et al. 2016). In addition, excessive production of mitochondrial ROS can activate the NLRP3 inflammasome through the TRX/TXNIP pathway. Moreover, inhibition of NF-κB in diabetic rats showed decreased levels of IL-1β and TNF-α in association with downregulation of TXNIP and NLRP3 in the kidney (Samra et al. 2016). Taken together, the ROS-TXNIP-NLRP3-NF-κB signaling pathway appears to be critical in promoting renal inflammation and subsequent kidney injury in diabetes (Fig. 7).

4.2.2 Novel Inflammation Inhibitors

MCC950, a diarylsulfonylurea-containing compound, is one of the most potent and highly specific small-molecule inhibitors of NLRP3 inflammasome. MCC950 prevents the NLRP3 conformational change and subsequent inflammasome formation by abrogating ASC oligomerization and thereby blocking the processing of IL-1β by caspase-1 (Coll et al. 2019). Anti-inflammatory and renoprotective effects of MCC950 have been demonstrated in various preclinical disease models including salt-sensitive hypertension, crystal-induced nephropathy (Krishnan et al. 2019) as well as in DKD (Zhang et al. 2019b). Indeed, MCC950 administration in *db/db* mice provided renoprotection as evidenced from attenuated renal fibrosis through suppression of pro-fibrotic markers such as TGF-β1, fibronectin, α-SMA, and collagen I as well as reduced thickening of the GBM, podocyte injury and albuminuria (Zhang et al. 2019b). The study reported that the renoprotective effects of MCC950 were achieved through downregulation of active caspase-1 and IL-1β by inhibiting the NLRP3/Caspase-1/IL-1β pathway in diabetic kidneys (Zhang et al. 2019b). More recently, anti-atherosclerotic effects of MCC950 have also been reported by our

group in a type 1 diabetic mouse model (Sharma et al. 2021). However, using the same dose and animal model, there was increased kidney injury with increased ROS formation and inflammation (Ostergaard et al. 2022). Thus, the renal effects of inflammasome inhibitors require further studies.

Tranilast, a tryptophan metabolite analog, appears to inhibit NLRP3 inflammasomes by impairing the assembly of endogenous NLRP3-ASC interaction. Use of tranilast has demonstrated significant preventive outcomes in gout arthritis and type 2 diabetic mouse models (Huang et al. 2018). Oral administration of this compound in diabetic mice showed improvement in reducing hyperglycemia and insulin resistance (Huang et al. 2018). In a clinical study, treatment of diabetic patients with tranilast was found to be associated with reduced urinary albumin and collagen IV excretion, suggesting the role of tranilast-driven inhibition of NLRP3 inflammasomes in early stage of DKD (Soma et al. 2006). Oridonin (Ori), a bioactive ent-kaurane diterpenoid, is reported to repress the release of inflammasome-dependent pro-inflammatory cytokines by inhibiting TLR4/NF-κB signaling pathways (Xu et al. 2009). Ori inhibits NLRP3 inflammasome activation by interacting with cysteine 279 of NLRP3 and thereby obliterating NLRP3-NEK7 interaction. Ori was found to be associated with reduced inflammation in experimental models of diabetes, peritonitis, and gout-related arthritis (He et al. 2018). In experimental diabetes, administration of Ori provided renoprotection by attenuating diabetes-induced renal injury and albuminuria via reduction in inflammation, including reduced infiltration of inflammatory cells in kidney tissues and decreased levels of pro-inflammatory cytokines, such as TNF-α, interleukin-6 (IL-6), IL-1β and MCP-1 through downregulation of TLR4 and inactivation of NF-κB pathways (Li et al. 2018). The experimental data suggest Ori as a potential therapeutic target in DKD and thus provides impetus for clinical studies in diabetic patients with nephropathy.

4.2.3 Chemokines and Cytokines

MCP-1, also known as monocyte chemoattractant C-C motif-ligand 2 (CCL2), is a pro-inflammatory chemokine and is upregulated in the kidneys as well as the urine of diabetic patients (Tashiro et al. 2002). *Mcp1* deletion in STZ-induced diabetic mice prevents the recruitment of macrophages to the glomeruli and reduces albuminuria (Chow et al. 2006). Pharmacological inhibition of MCP-1 signaling can be achieved with the so-called Spiegelmer emapticap pegol (NOX-E36), which is an anti-CCL2 L-enantiomeric RNA aptamer. Similar to the genetic studies, inhibition of MCP-1 by NOX-E36 in STZ-induced diabetic *ApoE* knockout mice resulted in attenuated albuminuria, and furthermore was shown to contribute to improvement of the glomerular filtration barrier by restoring the glomerular endothelial glycocalyx (Boels et al. 2017). Treatment of diabetic *db/db* mice with NOX-E36 resulted also in a reduced number of glomerular macrophages, associated with less distinct glomerulosclerosis and improved eGFR (Ninichuk et al. 2008). Furthermore, combination of NOX-E36 with the inhibitor NOX-A12 blocking another pro-inflammatory chemokine another pro-inflammatory chemokine, the stromal cell-derived factor 1 (SDF1), also known as C-X-C motif chemokine 12 (CXCL12),

even had additive protective effects on the kidney as it led to attenuated albuminuria and even higher eGFR levels (Darisipudi et al. 2011). Based on the promising findings of the preclinical studies, a phase 2 clinical study has been performed in type 2 diabetes patients with albuminuria who were treated with NOX-E36 for 12 weeks (NCT01547897). NOX-E36 treatment of diabetic patients resulted in reduced albuminuria, and thus confirmed its beneficial effects on the kidney, although no changes have been observed in the eGFR (Menne et al. 2017). The idea of targeting cytokines, such as IL-1β or IL-6, to inhibit their downstream signaling is based on their crucial role in the pathogenesis of DKD (reviewed in Donate-Correa et al. 2020). However, targeting of IL-1β by the specific inhibitor canakinumab resulted in less inflammation and lower rates of recurrent cardiovascular events in patients with previous infarction but also an increased number of fatal infections (CANTOS), and furthermore did not lead to clinically relevant improvement of the eGFR or albuminuria in patients with a previous myocardial infarction and CKD (Ridker et al. 2017; Ridker et al. 2018).

4.2.4 JAK-STAT

JAK signaling can be activated by ROS particularly under hyperglycemic conditions, and an increased JAK-STAT activity as well as expression has been observed in different mouse models of DKD (Brosius et al. 2016; Zhang et al. 2017). In diabetic 129S6 mice, overexpression of *Jak2* in podocytes significantly worsens DKD as these mice showed increased albuminuria, more glomerulosclerosis, and reduced podocyte density (Zhang et al. 2017). When these mice were treated with an inhibitor of both JAK-1 and JAK-2, LN3103801, this resulted in reduced JAK-STAT signaling as well as a reduction in albuminuria and mesangial expansion (Zhang et al. 2017). In *db/db* mice, inhibition of STAT3 acetylation, which is increased in mouse and human diabetic kidneys, was shown to reduce proteinuria and kidney injury (Brosius et al. 2016), suggesting that the JAK-STAT signaling is a promising target for the treatment of DKD in animal models. In humans, members of the JAK-STAT family have also been shown to be upregulated in glomerular and tubulointerstitial regions of the kidneys from patients with DKD (Berthier et al. 2009). Moreover, tubulointerstitial expression of JAK and STAT isoforms inversely correlates with the eGFR of these patients (Berthier et al. 2009). In a phase II clinical trial (NCT01683409), treatment with Baricitinib, a JAK-1/2 inhibitor, led to reduction of albuminuria and expression of renal inflammatory biomarkers in type 2 diabetes patients with DKD (Tuttle et al. 2018b). Thus, targeting the JAK-STAT pathway may be a potential novel therapeutic strategy for DKD.

4.2.5 Phosphodiesterase

Pentoxifylline is a phosphodiesterase inhibitor with anti-inflammatory and anti-fibrotic properties and has been shown to also have renoprotective properties in preclinical animal studies (Strutz et al. 2000; Davila-Esqueda and Martinez-Morales 2004; Lin et al. 2002). In STZ-induced diabetic Sprague-Dawley rats, pentoxifylline treatment reduced urinary albumin excretion and diminished oxidative stress in the kidney (Davila-Esqueda and Martinez-Morales 2004). In two smaller clinical trials with type 2 diabetes patients, administration of pentoxifylline for 4 months resulted

in decreased albuminuria (Navarro et al. 2003; Navarro et al. 2005). In another small study, pentoxifylline treatment of diabetic patients with advanced renal failure for 6 months also led to reduced urinary protein excretion (Navarro et al. 1999). This was confirmed in a longer clinical trial, in which type 2 diabetes patients with stage 3–4 CKD have received pentoxifylline for 2 years (PREDIAN) and showed a slowing of DKD progression as defined by attenuation of albuminuria and improvement of the eGFR decline (Navarro-Gonzalez et al. 2015). Notably, pentoxifylline treatment also results in a decrease of urinary TNF-α as well as MCP-1, which correlates with the urinary albumin excretion (Navarro et al. 2005; Navarro-Gonzalez et al. 2015; Lin et al. 2008), suggesting that its renoprotective effects are associated with its anti-inflammatory properties. This is supported by other studies, in which pentoxifylline treatment of patients with CKD resulted in a reduction of renal inflammatory markers (Goicoechea et al. 2010).

5 Outlook

For further identification of novel targets, the findings derived from single-cell sequencing in human and mouse diabetic and nondiabetic kidneys will help to identify gene patterns related to oxidative stress, inflammation, and fibrosis. This will form the basis for further evaluation of novel treatment targets in preclinical models of microvascular complications of diabetes, such as DKD, and ultimately for translation into the clinical context.

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Diabetic Peripheral Neuropathy

Johan Røikjer and Niels Ejskjaer

Contents

Abstract

Diabetic painless and painful peripheral neuropathy remains the most frequent complication of diabetes mellitus, but the pathophysiology remains undescribed, there are no robust clinical endpoints and no efficient treatment exists. This hampers good clinical practice, fruitful clinical research and successful pharmacological trials, necessary for the development of early detection, prevention and

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treatment. This chapter supplies an update on background and treatment of diabetic peripheral neuropathy. Goals and perspectives for future clinical and scientific approaches are also described.

Keywords

Diabetes · Neuropathy · Painful · Peripheral · Treatment

1 Introduction

Diabetic peripheral neuropathy remains the most frequent complication of diabetes mellitus affecting as much as 50% of all persons with diabetes (Feldman et al. 2019). Symmetrical sensory neuropathy of the lower extremities present as the most common pattern. Progress is often slow over many years, although some individuals experience faster and more severe courses. Despite the frequent occurrence, the causes of diabetic peripheral neuropathy are largely unknown, which is reflected in the fact that no disease-modifying treatments are available for preventing, treating or even halting the progression of the diabetic neuropathy (Røikjer et al. 2020a). Therefore, health care professionals are limited to more unselective preventive methods like optimizing glycaemic control, treating hypertension, lowering cholesterol levels and lifestyle interventions. The consequences can be dire, as neuropathy all too frequently leads to foot ulcers, amputations or intolerable neuropathic pain of the lower extremities, which is not only a burden for the individual, but also for the Health Care System as a whole (Røikjer et al. 2020b). Position statements on diabetic peripheral neuropathy like the Toronto consensus statement or the official statements from the American Diabetes Association have continuously brought attention to the subject, stressing the importance of early detection, prevention and sufficient screening (Tesfaye et al. 2011; Pop-Busui et al. 2017). Over the recent years, clinicians, clinical researchers and scientists have gradually provided better insights into the field, uncovering bits and pieces of the natural history and discovering potential targets for future drugs, moving us a little closer to solving the conundrum that is diabetic peripheral neuropathy.

2 Large- and Small Nerve Fibre Diabetic Neuropathy

Diabetic peripheral neuropathy is a diverse condition ranging from an asymptomatic state, a slowly progressing loss of sensation in the feet, to cases of rapid onset of invalidating symptoms including severe neuropathic pain. To truly understand the pathophysiological differences between these vastly different phenotypes of the same condition, one must first understand the differences between large- (Aα and Aβ) and small nerve fibres (Aδ and C).

As the name implies, the large nerve fibres are thick nerves (>5 μm in diameter), shed in fatty wrappings known as myelin. Like with electrical wires, this sheet helps

the nerves achieve faster conduction velocity, while periodic gaps in the isolation with a high density of ion-channels, known as nodes of Ranvier, help them achieve a relatively low activation threshold and high excitability. The large nerves carry signals from the peripheral towards the central nervous system, containing information on touch, vibration and proprioception. Damage towards these nerve fibres in diabetes is length dependent, and therefore firstly affects the feet and progresses proximally along the lower extremities symmetrically. At a later stage fingers and hands may be affected, again progressing proximally along the arms symmetrically.

The small nerve fibres, on the other hand, are thin fibres (<5 μm in diameter) with either a very thin myelin sheet or without myelin at all. This causes them to function a bit differently from large fibres, as their conduction velocity is markedly slower, especially that of the unmyelinated C-fibres, which causes the invoked sensation to be more diffuse, long-lasting and harder to specify. The small nerve fibres are responsible for the heat- and cold sensation as well as for the sensation of pain. Like with the large fibres, there also appears to be a length-dependent relationship in diabetes, which is why neuropathic pain caused by small nerve fibre damage is almost exclusively seen in the feet.

Large and small nerve fibres are both affected in diabetic peripheral neuropathy although the extent and onset may vary (Breiner et al. 2014). The main interest and focus has been on large nerve fibres with regard to screening and diagnosis of diabetic neuropathy. Large studies have linked large fibres to the development of diabetic foot ulcers, and their size and low activation threshold have enabled easy assessment utilizing conventional nerve conduction studies (Kong et al. 2008; Crawford et al. 2015). However, recent years of research have pointed to the importance of small nerve fibres, as they appear to be a relevant target for early detection of diabetic peripheral neuropathy, as the small nerve fibres in some studies appear to display detectable damage years in advance of large fibres (Dhage et al. 2021).

3 Early Detection of Diabetic Peripheral Neuropathy

Sensory loss of the feet may go completely undetected in diabetes, as there often are literally no symptoms. For many individuals, the development of diabetic peripheral neuropathy can therefore proceed completely unnoticed. Regular sensory testing is therefore the most important tool for diagnosing this condition. Unfortunately, unlike nephropathy or retinopathy, diabetic peripheral neuropathy is not easily screened for, as the condition lacks reliable clinical endpoints for early- or progressing disease (Røikjer et al. 2020a). Therefore, screening for diabetic peripheral neuropathy currently revolves around diagnosing loss of protective sensation, judged by the inability to feel vibration or light touch, and thereby only testing for large fibre function. In their most recent guidelines, however, the American Diabetes Association have included screening for small fibre neuropathy using either cold- and heat perception thresholds or pinprick as a clinical standard, thereby assessing temperature and pain sensations (Pop-Busui et al. 2017). Although this acknowledgement of the importance of assessing not only large- but also small nerve fibres is

a huge step towards early detection of diabetic peripheral neuropathy, the overriding issue of insensitive, unreproducible and inaccurate bedside tests for small nerve fibres remains. Sufficient sensitivity and specificity of methods for testing for cold- and heat perception and pinprick sensation are only achievable in dedicated neuropathy research centres, but remain poor and not applicable at a clinical level. The lack of sensitivity has become apparent in several large clinical trials, where the methods have continuously failed as robust clinical endpoints (Malik 2014). Due to this, the hunt for a sensitive and reproducible method for adequate assessment of small nerve fibres has begun. Amongst a number of promising methodologies, two have gained particular interest due to their diverse strengths, although clinical application is currently limited to a few specialized research sites.

The first of these two methods relies on quantification of the intra-epidermal nerve fibre density and is by many considered the gold standard for assessment of small nerve fibres (Lauria and Devigili 2007). The quantification of intra-epidermal nerve fibre density is achieved by performing a distal skin biopsy followed by staining and microscopy. This method is sufficiently sensitive and reproducible and has high inter-observer reliability. Recently clinical and basic science researchers have begun to utilize the biopsy for exploring the pathophysiology in addition to a mere quantification of nerve fibre density, which has led to interesting discoveries including the presence of axon-swellings and increased expression of, i.e., calcitonin-gene-related peptide and substance P in the diabetic small fibres (Karlsson et al. 2021; Albrecht et al. 2021). The growing interest in this method has led to the development of normative values for general use, although the highest reproducibility has been achieved by analysing the samples at the same lab (Lauria et al. 2010). While the method is very interesting and shows great promise, it does suffer several inherent weaknesses, including time-consumption, a need for specialized labs, advanced stainings and specialist competences. Also, the fact that you need a skin biopsy in people, who might already have poor wound healing and a risk of developing a diabetic foot ulcer, may limit a more general clinical utilization.

The second promising method is termed corneal confocal microscopy and utilizes the fact that the highest density of small nerve fibres in the human body is found in Bowman's capsule, located just below the superficial epithelium of the cornea in the eye (Malik et al. 2003). Using the multidimensional abilities of the confocal microscopy, the examiner can visualize and assess the nerve length and density of the small nerves in the eye, which have proven to be a surrogate marker for nerve damage elsewhere in the body. In contrast to skin biopsies this method is rapid and non-invasive and has similar abilities regarding reproducibility (Maddaloni and Sabatino 2016). While these are all in favour of corneal confocal microscopy, this method also has some limitations, including a need for highly specialized equipment and expertise, and the fact that many different diseases, like rheumatoid arthritis, may cause the method to indicate severe nerve degeneration without the person actually having diabetic peripheral neuropathy. Common for both of the above-mentioned methods are that both are limited to assessing the structural changes and damages of small nerve fibres, while do not describe function.

4 Pathophysiology of Diabetic Peripheral Neuropathy

Despite the high frequency of persons with diabetes and thus diabetic peripheral neuropathy, the exact mechanisms leading to the condition are not fully understood. Although there is a consensus that the toxic effect of hyperglycaemia plays an important role in developing the complication, the exact paths remain a mystery. In addition, recent years of research have made it clear that other factors besides hyperglycaemia should be considered when attempting to establish a description of the natural history.

Amongst several theories, the metabolic shift in glucose decomposition occurring when high levels of glucose saturate the hexosamine pathway remains the most generally accepted theory. This saturation leads to increased activity of the polyol pathway, which is linked to several issues within the cells. In this pathway, the rate-limiting first enzyme (aldose reductase) catalyses the generation of sorbitol from glucose, by oxidating nicotinamide adenine dinucleotide phosphate (NADPH) to $NADP^+$. Sorbitol is then further oxidized to fructose (by the sorbitol dehydrogenase), which ultimately results in reduced transformation of nicotinamide adenine dinucleotide (NAD^+) to NADH (Schreiber 2015). However, during periods of hyperglycaemia where the hexosamine pathway is saturated, the affinity of the aldose reductase for glucose is further increased, leading to the accumulation of sorbitol intracellularly (as sorbitol does not cross the cell membrane), which in turn induces osmotic stress. This was for many years thought to be the primary pathophysiological mechanism, but as more and more studies demonstrated insignificant concentrations of sorbitol within nerve cells from people with diabetes, a new theory has since been developed (Sheetz and King 2002; Baynes and Thorpe 1999; Oates 2002). This theory instead revolves around the issues related to the increasing turnover- and consumption rate of NADPH and NAD^+, which decreases the regeneration of glutathione, while simultaneously increasing the number of advanced glycation end products and activated protein kinase C isoforms (Schreiber 2015).

While oxidative stress might not be the primary pathophysiological mechanisms resulting from increased activity of the Polyol pathway, the overall impact of oxidative stress in the pathophysiology of diabetic peripheral neuropathy should not be neglected. While the Polyol pathway might be a contributor to increased oxidative stress, other conditions like autooxidation of glucose metabolites, increased formation of advanced glycation end products (especially intracellularly), increased expression of the advanced glycation end-product receptor and ligands, reduced or altered mitochondrial function and enhanced activation of protein kinase C isoforms might also contribute (Giacco and Brownlee 2010).

While the cellular mechanisms historically have been the most studied, diabetic peripheral neuropathy is after all classified as a microvascular complication alongside diabetic nephropathy and diabetic retinopathy, and reductions in peripheral, small vessel blood flow have been reported in many studies over the years. This reduction in blood flow (due to a reduced lumen) paired with increasing blood vessel wall thickness and hyalinization of the basal lamia leads to local nerve ischemia, which in turn results in progressive and irreversible nerve loss. Adding to this,

altered endothelial function with reduced endothelium-dependent- and endothelium-independent vasodilation has been reported in some studies of people with diabetic peripheral neuropathy, although some studies argue that the changes are not mediated by diabetic peripheral neuropathy, but more likely by diabetes per se. Furthermore, it also appears that hyperglycaemia alters the function of the Schwann cells, reducing their capacity to regenerate damages to the myelin sheaths, which might further contribute to the pathogenic condition within the nerves (Said et al. 2008).

Another more recent addition to the large array of potential causes of diabetic peripheral neuropathy is the role of systemic low-grade inflammation. However, the first link between these two conditions was actually described more than a century ago, where it was reported how high doses of sodium salicylate diminished glycosuria in people with "the mild diabetes" (presumably type 2 diabetes). In the more recent years, the topic has however been revived anew, as several studies have reported how increased levels of inflammation markers and acute-phase reactants like fibrinogen, C-reactive protein (CRP), interleukin (IL)-6, plasminogen activator inhibitor-1 (PAI-1), sialic acid and white cell counts correlate with incident type 2 diabetes and even some to the degree of nerve damage within these populations (Pop-Busui et al. 2016). The mechanism is thought to be mediated by an activation of several molecular pathways mediated by adipocyte-derived pro-inflammatory cytokines, including the activation of transcription factors nuclear factor-κB (NF-κB) and IκB kinase-β (IKKβ)/NF-κB axis. This would suggest that low-grade inflammation might predominantly be a factor in the pathogenesis of diabetic peripheral neuropathy in type 2 diabetes, but at the same time it has also been suggested that chronic inflammation contributes to the pathogenesis and development of atherosclerosis through multiple mechanisms including increased expression of vascular cell adhesion molecules on endothelial cells, enhanced recruitment of leukocytes, further release of pro-inflammatory cytokines, migration of macrophages and lipid oxidation (Libby et al. 2002). While most of the above-mentioned pathways predominantly lead to large vessel atherosclerosis, low-grade inflammation has also been linked to microvascular complications, with the Diabetes Control and Complications Trial (DCCT)/Epidemiology of Diabetes Intervention and Complications (EDIC) study cohort reporting that baseline E-selectin and fibrinogen levels were independently associated with the development of nephropathy in their large cohort of people with type 1 diabetes (Maria et al. 2008). Although the link between low-grade inflammation and diabetic peripheral neuropathy is not as robust as that of nephropathy, both animal and human studies have continuously reported increased levels of pro-inflammatory cytokines in people with diabetic peripheral neuropathy, with some studies even reporting even further increased levels in those with neuropathic pain (Doupis et al. 2009). In addition to this, a post-hoc, cross-sectional sample of almost 500 participants from the EURODIAB Prospective Complications Study found an independent association between serum HSP27 (which is associated with cytoprotection) and the presence of neuropathic symptoms, while an analysis with more than 1,000 participants from the population-based Cooperative Health Research in the Region of Augsburg (KORA) F4 study

reported a positive correlation between serum concentrations of inflammatory cytokines and different measurements of diabetic peripheral neuropathy (Gruden et al. 2008; Herder et al. 2013).

Lastly, the role of the sodium-potassium pump in the development of diabetic peripheral neuropathy should also briefly be mentioned, although decisive evidence is still missing. Several electrophysiological studies have however described decreased rates of recovery following maximal voluntary contractions in people with diabetes compared to people without, theorizing that the reduced threshold change and slower recovery in those with diabetes are likely to be secondary to sodium-potassium pump dysfunction (Krishnan et al. 2008).

Individual risk factors for developing diabetic peripheral neuropathy comprise age, alcohol, body mass index, ethnicity, sex, diabetes duration, diabetes type, glycaemic control, microangiopathic complications, waist circumference, hypertension and smoking. Some people with diabetes may develop diabetic neuropathy with one or no risk factors, while others do not in spite of a larger number of risk factors.

5 Treating Diabetic Peripheral Neuropathy

Describing the drugs registered as treatment for diabetic peripheral neuropathy is not a long-lasting task, as there are currently a grand total of zero drugs with that indication.

Previous studies attempting to halt or revert existing diabetic peripheral neuropathy have all failed, probably due to combinations of very short intervention periods and insufficient methods for assessing changes in nerve fibre function. The slowly progressing nature of diabetic peripheral neuropathy is the main challenge, as it is very challenging for a number of reasons to conduct a randomized clinical trial up to a decade, as it would be needed to achieve clinically relevant outcomes. Additionally, the inability to predict which individuals are in the greater risk of progression of diabetic peripheral neuropathy also hampers a successful conduction of clinical trials. Furthermore, a substantial number of participants are needed for a study to have sufficient power incurring very high costs. Therefore, our current understanding of the treatment of diabetic peripheral neuropathy is derived from clinical practice and observational studies, where prevention is preferred intervention.

In spite of all, the development of new and more sensitive methods for assessing the integrity of especially small nerve fibres gives hope for future studies. One example of this is a small study utilizing corneal confocal microscopy in people with type 1 diabetes receiving a kidney- and pancreas-transplant due to end-stage renal disease (Mehra et al. 2007). In this study, the authors detected nerve regeneration on the corneal imaging, indicating that regression of diabetic peripheral neuropathy may be possible, although the study should be interpreted with great caution, as the observations stem from a small cohort not dedicated to the particular study. The concept of reversible nerve damage has not only been investigated using corneal confocal microscopy, but also skin biopsies and other measurements of both large and small nerve damage have given evidence in this direction. Most recently it has

been described in a study from the German Diabetes Study cohort (Ziegler et al. 2021). In this study, the authors follow individuals with both type 1 and type 2 diabetes for 5 years, reporting the intriguing observation that initial alternations in nerve fibre integrity appear reversible to a clinically meaningful degree.

These observations, although initial and sparse, give hope for the development of future studies utilizing cutting-edge technologies in combination with mechanism-based treatments, which could ultimately result in a much-needed development of treatment of diabetic peripheral neuropathy.

6 Diabetic Foot Ulcers

Diabetic peripheral neuropathy is most often dominated by a complete lack of symptoms and is therefore often neglected by a huge part of the population with diabetes. As the peripheral neuropathy worsens, the condition will ultimately reach a stage where the individual will no longer have protective sensation in their feet, ultimately resulting in full anaesthesia. This means that they no longer feel it if they injure their feet, even large injury as a nail penetrating their forefoot, while walking barefooted. They will no longer sense if a small stone finds its way into their shoe, and they will not notice, if their new shoes are rubbing against their feet resulting in an unnoticed injury. This may result in the development of a diabetic foot ulcer and it happens in 19–34% of all individuals with diabetes. It is a condition associated with appointments with general practitioners, wound care, anti-microbial treatment, referrals to multidisciplinary teams at Diabetic Foot Care Centres, highly specialized treatment, off-loading, antibiotic treatment, revascularization – and in many cases hospitalization, continuous immobilization, amputation and a high risk of depression and pre-mature death (Røikjer et al. 2020b, 2021; Edmonds et al. 2021). At least 60% of diabetic foot ulcers are associated with severe diabetic peripheral neuropathy, with percentages as high as a staggering 90% in some reports (unpublished data) (Boulton et al. 2005). Furthermore, reduced proprioception and dysfunction of the autonomous nerve system results in enhanced local foot pressure and reduced sweat gland activity, increasing the biomechanical stress, and reducing the protective barrier of the skin at the same time. Adding to this, the neuropathy-induced reduction in blood vessel reactivity combined with atherosclerosis and a reduced large vessel peripheral blood flow further limits the regenerative abilities of the involved skin, increasing the risk of a broken skin barrier and reducing the ability to heal the foot ulcer at the same time.

Over the last decades, the treatment of diabetic foot ulcers has improved markedly, primarily due to the implementation of multidisciplinary teams at diabetic foot clinics, where endocrinologists, orthopaedic and vascular surgeons, podiatrists, specialized nurses, bandagists, radiologists, microbiologists and many more work together in a multifactorial approach to treat the actual foot ulcer while simultaneously preventing major amputations, hospital admissions and foot ulcer recurrence and at the same time attending to other diabetes complications (Musuuza et al. 2020). While the implementation has improved both amputation- and hospital admission-

rates, the mortality-rates after major amputation still rival most cancer diseases and the recurrence rate of diabetic foot ulcers has unfortunately not been affected as much as the other outcomes either, with roughly 40% still recurring within 1 year after healing and 60% recurring within 3 years (Røikjer et al. 2020b; Armstrong et al. 2017).

When assessing the individual risk factors associated with diabetic foot ulcer recurrence, diabetic peripheral neuropathy tops the list and some studies report increased morbidity and increased healing times of neuropathic foot ulcers compared to those primarily driven by vascular insufficiency (Armstrong et al. 2011, 2017). Other risk factors include peripheral arterial disease, osteomyelitis, depression, poorly regulated diabetes and inappropriate footwear, which in contrast to diabetic peripheral neuropathy might be reversed by a combination of antibiotics, vascular surgery, cognitive therapy and antidepressants, enhanced glycaemic control and patient education and development of good foot care habits supported by specialized footwear (Armstrong et al. 2011). However, reversing these risk factors is often easier said than done, as old habits die hard (i.e., walking around home barefooted or remembering to cut the nails and use moisturizing lotion), and can be even more challenging as many persons with diabetic foot ulcers are elderly and predominately men. Furthermore, the condition of the affected arteries and technical challenges may prevent revascularization and osteomyelitis is often difficult to treat even with state-of-the-art intravenous antibiotics and surgery. Current guidelines for treatment of osteomyelitis related to diabetic foot ulcers remain an area with very little evidence, where the scientific foundation is based on much broader evidence from osteomyelitis in general such as the OVIVA trial, without it being specific to diabetic foot ulcers or even people with diabetes (Vas et al. 2019; Li et al. 2019; Scarborough et al. 2019).

The most important risk factor with the highest impact for both development and recurrence of a diabetic foot ulcers remains diabetic peripheral neuropathy. There exists no treatment for prevention or treatment of this condition., Therefore the focus on diabetic foot ulcers relies on prevention of further ulceration by improving or neutralizing the other risk factors until specific efficient pharmacological interventions for diabetic neuropathy have been developed.

7 Pathophysiology of Neuropathic Pain in Diabetes

As is the case with non-painful diabetic peripheral neuropathy, not much is known about the pathogenesis of painful diabetic neuropathy, although this condition may affect as many as 20–30% of those with diabetic peripheral neuropathy in total. The relationship between painful- and painless diabetic peripheral neuropathy is not yet fully elucidated, and it remains an enigma, why some develop neuropathic pain and others do not. However, the presence of neuropathic pain without simultaneous peripheral sensory neuropathy is practically non-existing or very rare, which has led researchers and clinicians to conclude that pre-existing sensory neuropathy seems to be a pre-requisite for the development of painful diabetic neuropathy. It is however

unknown whether the two conditions purely co-exist, or a more distinct causative association exists.

Painful diabetic neuropathy may present as sharp, shooting, and deep pains of a burning, itching, or deep musculoskeletal sensation that can sometimes reach unbearable intensities. Fortunately, the condition is more frequently represented by mere uncomfortable symptoms like numbness, a feeling of walking on pins and needles, or a tingling sensation located deep within the musculoskeletal domains. The pain is often continuous or intermittent with spontaneous pain that may be accompanied by evoked pain caused by light touch or cold. The onset of the condition is often insidious, with symptoms gradually fluctuating over time, only for them to gradually disappear after years of disease.

The paradox of co-existing anaesthesia of the feet and intolerable pain is another conundrum yet to be solved. In recent years, researchers have been increasingly interested in distinct classifications and deep sensory profiling and phenotyping of those with painful diabetic neuropathy, as clinical observations have pointed out at least several distinct subgroups that might have vastly different courses and might benefit from different treatments (Finnerup et al. 2015). Despite the distinctiveness in each individual clinical presentation, the neurological examination is often very similar in those to painful- and painless diabetic peripheral neuropathy, which warrants the use of more sophisticated methods to truly classify each person. At an individual level, the person suffering moderate to severe painful diabetic neuropathy may experience a degree of physical disability, emerging depression and anxiety, insomnia and an overall poorer quality of life. It remains unclear whether the severity of diabetic peripheral neuropathy is greater when painful neuropathy is also present, although more and more evidence has recently appeared to support this claim in at least subgroups of those with pain. It should however be noted that severe diabetic peripheral neuropathy and painful neuropathy are not mutually exclusive, as painful neuropathy sometimes exists in people with mild or moderate diabetic peripheral neuropathy, which is also a distinct subgroup with potentially distinct pathophysiological abnormalities possibly different to those with severe neuropathy.

One of the many topics commonly discussed related to the different subgroups is related to whether pain is indeed evoked by injuries to the peripheral nerves or if the pain arises from signals from the spinal cord or even from altered connectivity in the brain itself. This interaction between peripheral damage and central sensibilization due to long-lasting pain is just one of many unknown interactions being studied in the field of pain research and could be one of several keys to uncovering other small parts of the largely unknown natural history of painful diabetic peripheral neuropathy. It has previously been demonstrated that primary afferents are sensitized in people with painful diabetic peripheral neuropathy, inducing dorsal horn hyperactivity and neuroplastic changes in central sensory neurons, which is further supported by the fact that allodynia is a common occurrence in people suffering from the condition (Chen and Pan 2002; Aslam et al. 2014). The causation of this sensitization is however less clear, although increased glutamate release from primary afferents in the spinal cord, enhanced spinal N-Methyl-D-aspartate receptor expression, and enhanced cAMP protein signaling have been proposed as plausible

mechanisms explaining the clinical observations and providing important targets for future treatment in subgroups of people with long-lasting pain (Schreiber 2015). In addition to alterations happening within the spinal cord, functional changes to the pain processing happening in the brain have also been demonstrated to be affected in painful diabetic peripheral neuropathy, although this field needs to be explored much further in order to better understand this extensive interchanging connectivity (Selvarajah et al. 2019).

In addition to the conundrum of central sensitization and altered brain plasticity, the current theories regarding why some persons with diabetic peripheral neuropathy experience pain while others do not include theories on channel sprouting, microglial activation and recently even increased low-grade inflammation (see Sect. 4).

Currently, the most accepted hypothesis of the development of neuropathic pain is related to disturbed action potentials produced by damaged nerve endings, which could be a direct result of changes in the composition or activity of ion-channels expressed in the peripheral nerve fibres leading to hyperexcitability. This results in a severe increase in signals being transmitted to the central nervous system, evoking a sensation of pain. The most widely recognized and studied ion-channels are the voltage-gated sodium channels (NaV), as they are the primary driving force behind the generation of action potentials and have been widely demonstrated in neuropathic pain models (Black et al. 1999; Blesneac et al. 2018). Amongst several candidates, NaV1.3, NaV1.7 and NaV1.8 have been of particular interest, as animal studies have indicated that these are upregulated in the dorsal root ganglia in rodent models of diabetes (Hong et al. 2004). For obvious reasons similar human studies are not available, but some studies have however reported an increase in nodal sodium currents in humans with diabetes and neuropathic pain when compared to those with painless diabetes peripheral neuropathy (Misawa et al. 2009). In addition to voltage-gated sodium channels, an altered function of calcium- (especially CaV3.2) and potassium channels have been proposed as parts of the overarching pathogenesis. Altered CaV3-2 function has been linked to the release of substance P and glutamate from sensory neurons, while altered function of the voltage-gated potassium channels affects resting membrane potential, thus inducing hyperexcitability (Kim et al. 2005; White and Zimmermann 1988).

Microglial activation has also previously been proposed as a contributing factor to neuropathic pain in people with diabetes. Under normal circumstances, microglia are activated during peripheral nerve injury, evoking a pro-inflammatory response with the production of several agents including cytokines, chemokines and nitric oxide. This response is usually abolished within less than 3 months, but from rodent models of type 1 diabetes it has been suggested that a persistent microglial activation is present, with some studies even linking it to the alterations happening to the NaV1.3 channels found within the dorsal root ganglia (Cheng et al. 2014).

The evidence for specific risk factors and their impact of the development for pain related to diabetic peripheral neuropathy remains sparse. As mentioned earlier, several individual risk factors have emerged for developing diabetic peripheral

neuropathy per se, but their impact on the risk of complication neuropathic pain remains unknown.

8 Conventional Treatment of Neuropathic Pain in Diabetes

The treatment of painful diabetic neuropathy is overall heavily influenced by the lacking knowledge of the pathophysiology of the condition. This means that both pharmacological and nonpharmacological treatment focus on non-specific relief or soothing of symptoms utilizing off-label use of drugs that were initially developed with greatly different applications in mind. Consequently, many of the applicable drugs are therefore inefficient in most persons suffering painful diabetic peripheral neuropathy, and often come with severe side effects and small therapeutic windows. Additionally, many individuals with diabetes and neuropathic pain also suffer other complications related to their diabetes, which means co-morbidities like ischemic heart disease or nephropathy must be taken into account when prescribing pain medication, further limiting available options. Due to these varying conditions, the treatment of painful diabetic peripheral neuropathy is a field in dire need for more personalized treatment regimes, which is only further stressed by the fact that polypharmacy is most often needed to achieve some level of pain relief.

Peripheral analgesics like paracetamol or non-steroidal anti-inflammatory drugs (NSAIDs) are rarely adequate for the treatment of neuropathic pain and are generally not recommended for chronic pain conditions like painful diabetic neuropathy. Instead, most guidelines encourage the use of tricyclic agents, serotonin-norepinephrine reuptake inhibitors and γ-aminobutyric acid analogues as first line treatment, often followed by slow-releasing opioids (i.e. Tapentadol ER) and topical treatment (Røikjer et al. 2020a; Spallone 2012; Javed et al. 2015).

Tricyclic agents are multimodal action drugs that block the serotonin and noradrenaline reuptake, while also inhibit anticholinergic receptors. In addition to this, several agents from this drug class also act as partial sodium-channel blockers, although the full mechanism and impact of this effect are yet to be described in detail. Drugs in this drug class include, but are not limited to, amitriptyline, clomipramine, desipramine, imipramine and nortriptyline, where amitriptyline is the most used and best examined option in people with diabetes. The most impactful study regarding the efficiency and safety of amitriptyline is the most recent review from the Cochrane Collaboration (Saarto and Wiffen 2007). In this review and meta-analysis (with 5 studies included) treatment with amitriptyline seemed to provide an impressively low number of people needed to treat (1.3 persons) in order to achieve a successful reduction in pain intensity. However, the use of tricyclic agents in doses used in the studies from this review is more often than not associated with significant and severe side effects that massively limit their use in clinical practice, probably increasing the reported number needed to treat markedly when applied to the average person with painful diabetic peripheral neuropathy. Side effects include, but are not limited to, anticholinergic side effects like xerostomia, urinary retention, obstipation and difficulties with accommodation as well as cardiac- and psychological side

effects like cardiac arrhythmias, somnolence, fatigue, dizziness and insomnia (Griebeler et al. 2014). The use of tricyclic agents may also be associated with increases in body weight, which is often already a challenge, not least in people with type 2 diabetes.

Serotonin-norepinephrine reuptake inhibitors are, as the name implies, agents that inhibit reuptake of both serotonin and norepinephrine. The most used drug from this category is by far duloxetine (and to a lesser extend venlafaxine). The Cochrane Collaboration also evaluated duloxetine in a relatively recent meta-analysis, concluding that a daily dosage of 60 mg duloxetine would result in a more than 50% pain reduction in one out of five persons with neuropathic pain (Lunn et al. 2014). The most common side effects related to the usage of serotonin-norepinephrine reuptake inhibitors are nausea, somnolence, dizziness, constipation and dyspepsia, but more severe side effects like Steven-Johnson syndrome, glaucoma or cardiac arrhythmias have also been reported. However, in contrast to tricyclic agents, SNRIs do not cause weight gain (Griebeler et al. 2014). Duloxetine is one of only four drugs currently approved for usage in painful neuropathy by the US Food and Drug Administration.

γ-Aminobutyric acid analogues is the final drug class that is commonly used as first line treatment for painful diabetic peripheral neuropathy. The class includes several different options, with gabapentin and pregabalin being the two most used agents. The mechanisms of action for these drugs include calcium channel modulation through inhibition of the a2δ subunit. Pregabalin is probably the most extensively studied drug in painful diabetic neuropathy, with the most recent meta-analysis including more than 9 trials with more than a total of 2,000 participants (Zhang et al. 2015). From this analysis, the number needed to treat in order to achieve a more than 50% pain reduction appears to be around 7.7 persons with painful diabetic neuropathy (Zhang et al. 2015). A similar analysis made for gabapentin reported a comparable number needed to treat of 6.6 indicating a similar effect to that of pregabalin (Gaskell et al. 2016). Common side effects for γ-aminobutyric acid analogues include somnolence, dizziness, headaches and elevated liver enzymes, as well as significant increases in body weight (Griebeler et al. 2014). Like duloxetine, pregabalin is also approved by the US Food and Drug Administration for the treatment of neuropathic pain.

While most guidelines emphasize the importance of using these first line treatments, the number of larger clinical trials directly comparing the safety and efficiency of them is relatively low. One mentionable, ongoing trial is the United Kingdom-based, OPTION-DM trial, that is comparing efficiency and safety of amitriptyline, duloxetine and pregabalin in a large cohort recruited from 8 hospitals and 80 general practices (Selvarajah et al. 2018). Another mentionable (completed) study is the COMBO-DM trial, that compared duloxetine 120 mg or pregabalin 600 mg daily with a combination of the two in half dose (60 mg duloxetine and 300 mg pregabalin), where the authors found no significant difference between the different treatment-regimes (Tesfaye et al. 2013).

Other drug classes used in the treatment of painful diabetic peripheral neuropathy include sodium channel blockers and opioids with extended releases. These drug

classes have been tested in several clinical trials with varying results (Røikjer et al. 2020a). The sodium channel blockers (mainly carbamazepine and the successor oxcarbazepine) have been tested in only a few larger clinical trials with a number needed to treat to achieve a more than 50% reduction in neuropathic pain around 6 persons (Zhou et al. 2017). More interestingly though, one trial evaluated oxcarbazepine as sort of a personalized treatment option for specific subgroups of people with painful diabetic neuropathy, as an altered effect and/or composition of voltage-gated sodium channels have been proposed as one of several pathophysiological mechanisms that could potentially result in neuropathic pain (Vollert et al. 2017; Demant et al. 2014). Subgrouping their study populations, the authors of that study managed to reduce the number needed to treat to achieve more than 50% pain reduction from 6.9 in the total population to only 3.9 in the sub-grouped population, thus for the first time indicating that personalized, mechanism-based treatment is what we must strive for in the future (Demant et al. 2014). Unfortunately, carbamazepine has been withdrawn from clinical trials due to severe side effects. These include, but are not limited to, dizziness, somnolence, vomiting and agitation (Dogra et al. 2005).

Opioids are well-known and well-examined pain medication that is used in a large variety of different painful conditions with great effect. The drugs enact their pain-relieving effect by interacting with the μ, δ, or κ opioid receptors. Although well-examined in many conditions, the effect of opioids on neuropathic pain conditions such as painful diabetic peripheral neuropathy remains controversial, as no larger clinical trials have established their effect in these conditions (Røikjer et al. 2020a). Adding to this, long-term treatment with opioids is generally not recommended, as it is associated with opioid dependency, abuse and the need for continuously increasing doses due to a decreasing effect over time. Furthermore, side effects like nausea, dizziness, constipation, itching and orthostatic hypotension are also very common with increasing prevalence amongst elderly persons (Benyamin et al. 2008). Despite this, one opioid (Tapentadol extended release) has been approved for the treatment of painful diabetic peripheral neuropathy by the US Food and Drug Administration. Tapentadol has a unique dual action that combines norepinephrine reuptake inhibition with a weak affinity for the μ-opioid receptor. This way, the mechanism of the drug reduces the prevalence and severity of the classic side effects otherwise associated with opioid use. Tapentadol extended release has been studied in several large trials, one of which was a 3-week, open label, phase III trial including almost 600 persons with painful diabetic peripheral neuropathy with a poor response to first line treatment (Schwartz et al. 2011). In this study, the authors found a significant reduction in pain when compared to placebo, which was only further supported in the 12-week follow-up, where more than half experienced more than 30% reduction in pain intensity (Schwartz et al. 2011).

Other treatments include topical treatment with, i.e., capsaicin, clonidine, isosorbide dinitrate, lidocaine or botulinum toxin A, although these options are restricted to the more treatment refractory cases (Røikjer et al. 2020a). Endocannabinoids may be used off-label, but there exists no clinical evidence and the efficacy remains unproven.

Finally, adequate or acceptable pain relief is often not achievable with the above-mentioned options, administered solely or in combinations, in a number of individuals with painful diabetic peripheral neuropathy. Therefore nonpharmacological alternatives are often applied, including transcutaneous electrical nerve stimulation, spinal cord stimulation, acupuncture and mindfulness, although it should be stressed that no high-quality evidence supports effectiveness of these interventions (Røikjer et al. 2020a).

9 Pathogenic Treatment of Neuropathic Pain in Diabetes

Pathogenic treatment is pharmacological treatment thought to act directly on some of the proposed mechanisms behind the development of diabetic peripheral neuropathy (Røikjer et al. 2020a). One example of such a treatment is the possible intervention on a specific subset of voltage-gated sodium channels as briefly mentioned earlier. Pathogenic treatment is theoretically superior to current standard of care, as the effect could be greater, while the side effects are believed to be less severe. Unfortunately, no pathogenic treatment is currently approved for the treatment of painful diabetic peripheral neuropathy in a clinical setting, as most aspects of the pathophysiology remain a mystery. However, several different drugs have been proposed as options in subgroups of individuals with neuropathic pain, although the number of studies conducted to support this claim remains sparse. Amongst several options, α-lipoic acid, C-peptide, benfotiamine and aldose-reductase inhibitors are some of the more interesting options (Javed et al. 2015).

α-Lipoic acid is an antioxidant with the purpose of reducing the impact of oxidative stress (Vincent et al. 2011). The rationale behind this treatment builds on several animal studies detecting an increase in the production of free radicals and a deficient antioxidant mechanism in rodent models of diabetes. α-Lipoic acid is available as oral or intravenous treatment, the latter having been the most studied. Several individual studies have provided promising results, but generally build on inconsistent or poor methodological quality and a definitive proof of the efficiency of the treatment is still pending (Han et al. 2012).

Aldose-reductase inhibitors are drugs interacting with a key enzyme in the polyol pathway, which (as described earlier) is one of the oldest and most generally accepted contributors to the development of diabetic complications including diabetic peripheral neuropathy (Chalk et al. 2007). Unfortunately, most studies have provided mixed results or have been stopped due to severe side effects. Amongst several aldose-reductase inhibitors, the most mentionable drug is epalrestat, which was used in an extensive 3-year trial, indicating possible potential benefits attempting to halt the progression of diabetic peripheral neuropathy and onset of neuropathic pain, but the evidence remains inconclusive (Hotta et al. 2006).

Benfotiamine is a lipid-soluble derivate of vitamin B1 that allows penetration of nerve cell membranes (Stracke et al. 1996). The rationale behind its use is the potential protection against and clearance of the accumulated advanced glycation

end products. Benfotiamine has not been studied very extensively in clinical trials, and its usage remains mostly theoretical.

C-peptide is an amino acid component of proinsulin, which is well-known from the clinic as it is traditionally used to evaluate endogenous insulin-production and differentiation of diabetes type. The idea behind using it as an actual treatment comes from animal models, where it has been suggested that C-peptides may stimulate the Na^+/K^+-ATPase, thus reversing its inactivity (Ekberg et al. 2007). While the theory is interesting, larger clinical studies have not been performed.

As described earlier, targeting low-grade inflammation (via modulation of the IKKβ/NF-κB pathway) or the altered sensitization in the spinal cord could also be viable options going forward, although much work is needed before large-scale clinical trials can be initiated.

Lastly, the inhibition of axonal voltage-gated sodium channel (like NaV 1.7 or 1.8) should be mentioned. The idea behind this drug target has been derived not only from animal models of especially type 1 diabetes, but also from rare genetic mutations found in humans, where super excitability of the nerve membrane due to mutations in the mentioned sodium channels results in continuous and rapid action potentials and thus activation of the pain system. As mentioned earlier, unselective sodium-channel blockers like oxcarbazepine already exist, but their selectiveness means that their safety profile is often unacceptable. The concentrations probably needed to provide sufficient effects would be toxic in humans. Unfortunately, it has not yet been possible to produce selective sodium channel blockers, as the current options appear to be either too unselective or have insufficient effect.

Ultimately, none of the above-mentioned drugs and none of the drugs used for diabetic peripheral neuropathy have been investigated sufficiently to produce any form of significant evidence level for usage in painful diabetic peripheral neuropathy. Overall, drugs targeting the specific pathophysiological mechanisms in distinct subgroups of individuals with painful diabetic neuropathy are a viable option to achieve both adequate and side effect-free treatment for this incapacitating complication.

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Part III

Lifestyle Modifications and Future Treatments

Weight Loss Strategies

Susan B. Roberts, Stephen Anton, and Maria C. Dao

Contents

Abstract

Lifestyle interventions for weight loss combine support for changing diet and physical activity with weight management education and are considered the first line treatment for obesity. A variety of diet-focused interventions including time-restricted eating are also increasingly being promoted for weight management. This chapter reviews different types of interventions for weight management, their underlying health behavior change models, and effectiveness to date in

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randomized trials. The results justify increasing efforts to improve program effectiveness generally, and to personalize interventions to support long-term adherence. The high prevalence of obesity worldwide, combined with the known increase in risk of non-communicable diseases with duration of excess weight, provides a compelling justification for routine delivery of effective weight management interventions in the community and in clinical care.

Keywords

Behavioral interventions · Lifestyle interventions · Precision nutrition · Weight loss

1 Introduction

Obesity is a major underlying cause of most non-communicable diseases including type 2 diabetes, cardiovascular disease, and several cancers (Pi-Sunyer 2009; Kopelman 2000). Obesity is also a strong independent risk factor for dementia, Alzheimer's disease, and disability (Xu et al. 2011; Launer et al. 1994) and reduces workforce productivity and quality of life (Goettler et al. 2017). During the past four decades the prevalence of obesity has risen in every region of the world (World Health Organization 2021). Today, 42.4% of adult Americans have obesity (Center for Disease Control and Prevention 2018) and 39% of adults worldwide have overweight or obesity (World Health Organization 2021), which is causing an unsustainable global burden of non-communicable diseases and increased health care costs. The prevalence of obesity has increased despite widespread awareness of the medical problems caused by obesity and a high percentage of the adult population trying to lose weight each year (Snook et al. 2017). This chapter reviews lifestyle-based approaches to weight management that are in current use.

2 Energy Intake and Energy Expenditure in Different Approaches of Weight Loss

Obesity results from prolonged imbalance between energy intake and energy expenditure, leading to excess accumulation of fat mass. The classical data for the energy cost of weight gain is 7,000 kcal of excess energy intake over total energy expenditure to produce a 1 kg weight gain (Marzola et al. 2013). This numerical figure (7,000 kcal) mostly addresses the net energy content of new tissue, however, it is also necessary to take into account the increase in energy expenditure associated with weight gain. Energy expenditure can be estimated through established equations based on a person's height, weight, sex, and activity level or can be directly through the research method called doubly labeled water (Speakman et al. 2021).

To lose weight requires reducing energy intake below energy expenditure, so that body fat can be mobilized and used as an energy source. After weight has been lost, energy requirements are lower than before weight loss, at least in part because most components of energy expenditure are weight-dependent. For this reason, smaller bodies typically use less energy than larger bodies. These fundamental principles of energy regulation indicate that a negative energy balance is essential for weight loss, and after weight is lost, energy intake has to remain decreased relative to energy intake levels prior to weight loss, otherwise weight will be regained (Ebbeling et al. 2007; Greenway 2015; Blomain et al. 2013; Faulconbridge and Hayes 2011; Nicolaidis 2019).

A long-running area of controversy in obesity research is whether energy requirements decrease after weight loss more than expected for the amount and quality of weight that is lost (i.e., taking into account changes in fat-free mass and fat mass) (MacLean et al. 2011; Martins et al. 2020). Support for the concept that energy expenditure in the post-weight loss state is lower than expected for the degree of weight loss is based on those studies reporting decreases in total daily energy expenditure (Redman et al. 2009; Rosenbaum et al. 2008; Ravussin et al. 2015), energy expenditure for physical activity (Redman et al. 2009; Rosenbaum et al. 2008; Ravussin et al. 2015), or, in some studies also in resting energy expenditure (Rosenbaum et al. 2008; Van Gemert et al. 1998) after mathematical adjustment for the effects of a decrease in body fat-free mass and fat mass.

In contrast, other studies have reported much smaller changes in energy expenditure following weight loss – and in some cases no changes at all. The randomized CALERIE trial of calorie restriction in 220 individuals with a body mass index (BMI) in the normal-to-overweight range (Ravussin et al. 2015) measured changes in total daily energy expenditure over 2 years in response to calorie restriction. At the 2 year follow-up assessment, there was no significant reduction in resting energy expenditure among participants in the calorie restriction group compared to assessment-only Controls (who did not lose weight) after accounting for changes in fat-free mass and fat mass. In addition, there was only a 7.3% mean decrease in total daily energy expenditure for a mean weight loss of 10.4%. Similarly, in a study of gastric bypass patients, there was no significant decrease in body-composition adjusted data for total daily energy expenditure and resting energy expenditure in patients losing an average of 53 kg body weight over 14 month (Das et al. 2003). Other groups have reported no difference in total daily energy expenditure or components of energy expenditure including resting energy expenditure between individuals in the weight-reduced state and BMI-matched individuals who had not lost weight (Amatruda et al. 1993). Combined, these conflicting data do not categorically support or deny the existence of mechanisms resulting in metabolic efficiency following weight loss that can contribute to weight regain. Thus, further research is needed to better understand the extent to which metabolic adaptation exists after weight loss and contributes to weight regain.

3 Lifestyle Interventions for Weight Management

Lifestyle interventions aim to facilitate weight loss by supporting participants efforts to achieve negative energy balance (i.e., low energy intake relative to total daily energy expenditure) with behavior change support and synchronized education based on models of health behavior change. Most lifestyle interventions for weight management are today informed by multiple theories of health behavior change (Ajzen 1985; Palmeira et al. 2007; Glanz et al. 1990; Glanz and Bishop 2010; Baranowski et al. 2003; Locke and Latham 1990; Smith et al. 2000). In particular, Social Cognitive Theory (SCT) (Bandura 1986) is a strong underpinning for many behavioral interventions because its central principle that an individual's behavior exists in reciprocal relationships with personal factors (i.e., cognitions, emotions) and environmental factors is consistent with a personalized approach to weight management. Interventions based on SCT have consistently been found to support the initiation of behavioral changes in weight loss interventions (Adhikari et al. 2018).

We recently proposed an updated version of SCT that includes a biological factor as an important component of reciprocal determinism which interacts with behavioral, personal factors and environmental factors (Anton et al. 2021) (Fig. 1). This revision was based on the increasingly compelling evidence that weight management is not simply a matter of willpower or personal factors, such as thoughts and emotions, and that hunger and satiety levels can have significant influences on energy intake, programmatic adherence, and success of intentional weight loss and weight loss maintenance (Pasman et al. 1999; Wing et al. 2008; Batra et al. 2013; Deckersbach et al. 2014).

3.1 Traditional Lifestyle Interventions

Traditional lifestyle interventions such as the Diabetes Prevention Program (DPP) (Diabetes Prevention and Support Center 2018) support standard program components such as goal setting, planning, development of self-regulation skills (e.g., self-monitoring), stimulus control, and relapse recovery, flexible eating restraint and increasing self-efficacy through incremental accomplishments. Goal setting and accountability are prioritized, with the result that daily logging of food and activity are core programmatic requirements. Operationally, such interventions are implemented in group or individual meetings with trained counselors. Historically these meetings have been in person, though two recent studies by our group indicate that video conference meetings have similar levels of effectiveness (Das et al. 2017; Dao et al. 2020) and counselors agree on daily goals for energy intake (typically reducing energy intake by 500–1,000 kcal/day to achieve weight losses of 1–2 lbs/week), reducing fat and or energy density, and increased physical activity is expected. The daily logs prepared by participants are given to the counselor weekly, who reviews them and provides feedback. There are also educational units and handouts on a variety of topics that are provided during meetings to support success.

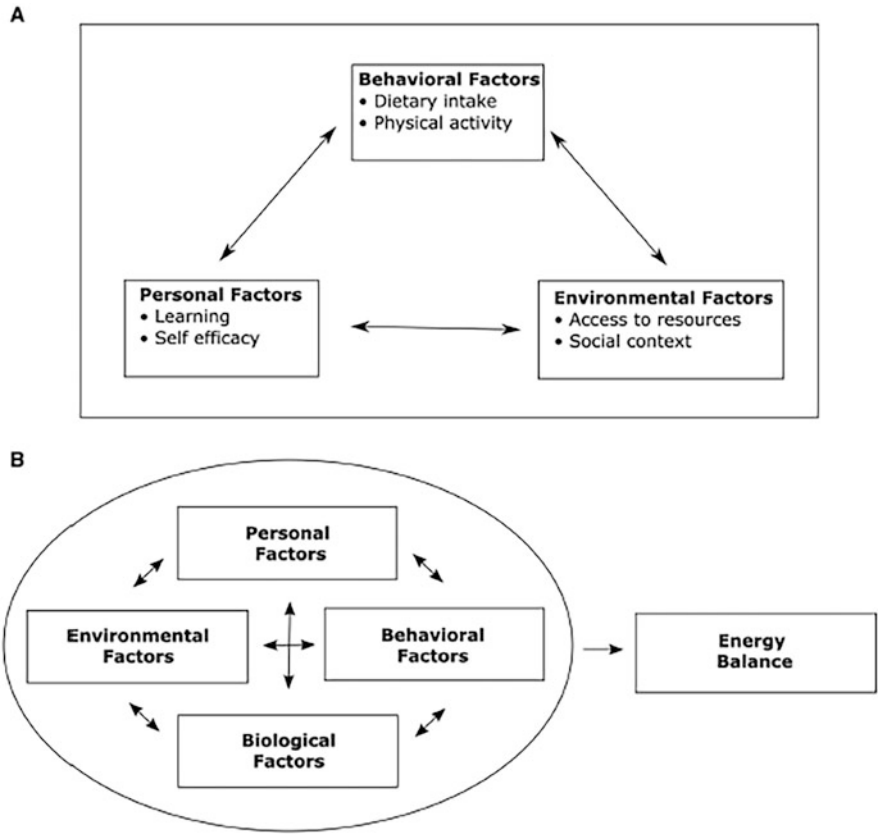

Fig. 1 Traditional and recently revised health behavior change models. (**a**) Traditional model of reciprocal determinism in social cognitive theory. (**b**) New model of reciprocal determinism illustrating the multiple and interactive factors affecting energy balance. Reprinted with permission from (Anton et al. 2021)

Such lifestyle interventions can produce weight reductions of 5–10% of baseline weight over the course of 4–6 months when conducted in intensive research studies (Expert Panel Members et al. 2014; Force 2018; Wadden et al. 2020; Heymsfield and Wadden 2017). However, national scaling of DPP in the USA achieved only 3.4% mean weight loss (and high drop-out) (Rehm et al. 2017) with estimated program costs of $400 and $278 total health care cost savings over 3 years (Vojta et al. 2013; Alva et al. 2017). This mean weight loss compares unfavorably with the $\geq$5% weight loss identified to have active health benefits (Jensen et al. 2013), indicating that research is needed to continue to improve lifestyle interventions both from the perspective of sustainable effectiveness and to minimize participant drop-out.

3.2 Alternative Lifestyle Interventions

We developed an alternative lifestyle intervention, Healthy Weight for Living (HWL), to address the high participant burden and low effectiveness of scaled DPP interventions. The intervention differs in several ways from DPP (Fig. 2). Like DPP, HWL is informed by multiple theories of health behavior change, but uses the revision of SCT noted above that includes a biological component (Ajzen 1985; Palmeira et al. 2007; Glanz et al. 1990; Glanz and Bishop 2010; Baranowski et al. 2003; Locke and Latham 1990; Smith et al. 2000) to support standard program components including goal setting, planning, development of self-regulation skills, stimulus control, and increasing self-efficacy. The inclusion of a biological component in SCT results in HWL prioritizing eating behavior changes to reduce the frequency and intensity of hunger and food cravings concomitantly with building healthier food preferences. Based on the studies of the acute effects of dietary

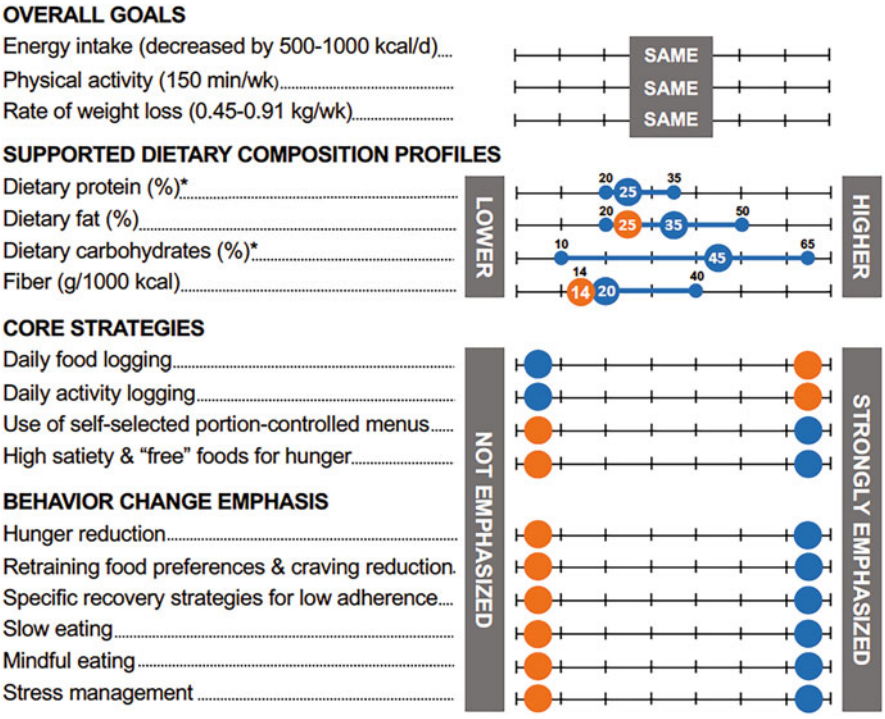

Fig. 2 Summary of differences in implementation goals and strategies used in Healthy Weight for Living (HWL, denoted by ●) versus the modified Diabetes Prevention Program (m-DPP, denoted by ●). For Supported Dietary Composition Profiles, default targets for HWL and m-DPP are listed in white within blue or orange circles, respectively; supported ranges for HWL are indicated by blue lines (●—●), with values listed in black at upper and lower limits. An asterisk (*) denotes that a default target is not specified by DPP for the corresponding dietary component. Reprinted with permission from (Das et al. 2021)

composition on hunger and satiety, multiple dietary composition options are provided in HWL (high protein and fiber, low glycemic index carbohydrates, low glycemic load, and low energy density (Roberts 2000; Eisenstein et al. 2002; Yao and Roberts 2001; Howarth et al. 2001; Gilhooly et al. 2008)). These different composition options are used individually and in combination to allow for flexible dietary composition recommendations ranging from low-carbohydrate to low-fat and vegan diets.

Thus, HWL contrasts with DPP, which specifically supports a low-fat and low-energy density prescription (25% of energy from fat with low energy density), by emphasizing flexible eating patterns with an emphasis on a Mediterranean-style, low glycemic load, and high fiber composition. A second way that the health behavior change model for HWL differs from DPP is that it prioritizes the development of *intrinsic* motivation (Ryan and Deci 2000a) for the behavior changes that cause negative energy balance. Intrinsic motivation is defined as doing something for its inherent satisfactions rather than from separable consequences (Ryan and Deci 2000b). Thus, the health behavior model for HWL prioritizes developing direct motivation for the food intake and activity behavior changes that cause weight loss, rather than motivation for weight loss with changes in food and activity viewed as necessary to achieve weight loss. This approach contrasts with DPP, which builds extrinsic motivation for weight loss, and dietary and physical activity behaviors are positioned for their anticipated effects on weight.

Operationally, HWL is similar to DPP in that has educational units and handouts on a variety of topics that aim to support success related to the specific programmatic goals and behavior changes it prioritizes. However, instead of asking participants to log all daily food, HWL provides portion-controlled self-selection menus tailed to the anticipated energy requirements of the participants that embed its flexible dietary composition parameters; simple menu adherence tracking allows for accountability without the need for burdensome daily food logging. The menu-centered approach combined with specific stimulus-control education on hunger also has the advantage of greater behavior change specificity, which itself may support programmatic adherence (McNabb et al. 1993; Shintani et al. 1991; Patterson et al. 1997), and addresses craving reduction through menu repetition to entrain healthy food habits (Sallis et al. 2006; Story et al. 2008; Swinburn et al. 1999; Brug et al. 2006). In addition, HWL education units allow for flexible meal timing options including modified fasting and time-restricted eating (discussed further below). An additional operational difference from DPP is that HWL introduces behavior change for physical activity after establishing changes in food patterns.

In terms of the effectiveness of HWL, a recent trial indicated comparable effectiveness to DPP when both interventions were matched for contact time with counselors (Das et al. 2021). Such findings suggest that HWL can serve as a potential alternative intervention for individuals who are not able or willing to do the burdensome daily food and activity logging required in traditional lifestyle interventions, such as DPP.

4 Time-Restricted Eating Patterns

Time-restricted eating (TRE) is an alternate proposed approach to weight management that reduces the need for intensive behavioral support because its primary focus is on restricting the periods of time in which food is consumed rather than restricting the types of foods or specific macronutrients consumed. In addition to its theoretical ease of implementation which could lead to reductions in energy intake through restricting the time when food can be eaten, TRE has been proposed to have the potential for metabolic benefits over and above those attributable to calorie restriction alone, including preservation of muscle and improved insulin sensitivity (Anton et al. 2018). An example TRE regimen would involve eating during specific periods of the day (e.g., during an 8-h time span). Time-restricted eating falls within the broader category of intermittent fasting, which also includes alternate day fasting regimens that completely or substantially restrict food on some days of the week (Heilbronn et al. 2005).

Time-restricted feeding ameliorates dysmetabolism in mice with diet-induced obesity and also provides improvements in cardiometabolic risk factors and protection against obesity in humans (Anton et al. 2018; de Cabo and Mattson 2019). Clinical studies to date, however, have provided no clear indication on whether time-restricted eating results in equivalent weight loss compared to traditional lifestyle interventions, which focus on calorie restriction independent of meal timing (Aksungar et al. 2017; Rynders et al. 2019; Phillips et al. 2021). Furthermore, the apparent simplicity of TRE may be deceptive, because many participants appear to not understand the requirements of this eating pattern even when provided with specific instructions (Lee et al. 2020). Reported specific challenges to adherence include lack of understanding that no-calorie containing snacks are allowed during fasting periods, as well as confusing low-calorie with no-calorie items.

5 Diet Composition Focused Interventions

Dietary patterns with the types of ultra-processed foods that are widely available worldwide readily cause overeating and weight gain (Hall et al. 2019), indicating that diet composition has the potential to significantly impact weight management. A wide range of weight loss programs exist that focus primarily on restricting different foods or categories of foods. An underlying principle that might support the effectiveness of this kind of approach is that dietary variety is a strong driver of food consumption so restricting the variety of unhealthy foods that can be consumed may facilitate reduced energy intake (McCrory et al. 1999).

Low-carbohydrate diets in particular may also facilitate reduced energy intake through suppression of appetite (Gibson et al. 2015). A recent systematic review of 16 trials (Anton et al. 2017) examined the 6- and 12-month effects of a range of different restrictive dietary practices including low-carbohydrate, low-fat, conventional diet recommendations such as Dietary Approaches to Stop Hypertension, low glycemic index diets, and Mediterranean diets. Other reviews have reported

beneficial effects of other dietary patterns for weight loss, including low glycemic index diets loss (Zafar et al. 2019) and vegan diets (Huang et al. 2016).

Reports that multiple different diet-restricting practices have beneficial effects on weight raise the question of whether the effects of different dietary parameters are substitutive (i.e., similar magnitude of effects can be obtained with several different types of diets) or additive (i.e., the effects of different dietary features on changes in body weight are additive (Urban et al. 2014). In a provided-food inpatient feeding studies of ad libitum dietary choices and energy intake from a self-selection menu, there were additive effects on energy intake for percent energy from protein (negatively associated with energy intake), and dietary variety, glycemic index, percent energy from liquid sources and energy density (positively associated with energy intake), supporting the view that at least to some extent the effects of different dietary factors have cumulative effects on energy intake and body weight change (Urban et al. 2014). The science of additive effects of different dietary factors on energy intake is however at an early stage, and further research in this area is needed.

6 Effects of Age on Weight Management

Older adults with obesity are at particular risk of diseases and conditions associated with aging including all the major non-communicable diseases, cognitive decline and dementia, obstructive sleep apnea, sensory impairments (age-related macular degeneration, cataracts, diabetic retinopathy and hearing loss), urinary incontinence, and frailty (Jensen et al. 2013; Roberts et al. 2021; GBD Obesity Collaborators 2017; Yamaoka et al. 2019; Subak et al. 2009; Franklin and Lindberg 2015; Amiri et al. 2020; Silverwood et al. 2015; Beydoun et al. 2008; Crow et al. 2019). Fortunately, a behavioral weight loss intervention is an effective first line treatment of several of these age-related conditions in individuals with obesity, including urinary incontinence and sleep apnea (Subak et al. 2005; Mitchell et al. 2014). Furthermore, a mean weight loss of 10% has been reported to achieve remission of type 2 diabetes in 50% of cases when implemented with within 7 years of onset, providing a remarkable example of the potential for lifestyle-based weight loss interventions to impact age-related metabolic disease conditions more effectively than current medications (Lean et al. 2018).

The question of whether weight loss interventions can be effective in older adults is a topic of increasing interest. Energy requirements decline in old age (Roberts et al. 2021) which in theory could make weight loss and prevention of weight regain more challenging. As illustrated in Fig. 3, some studies have shown that lifestyle interventions have similar levels of effectiveness for weight loss in adults >60 years of age compared to those who are 30–60 years old, and are more effective in older adults than in adults aged 20–30 years (Das et al. 2017). These results indicate that old age does not need to be a barrier to healthy weight management using a lifestyle intervention approach, despite declining energy requirements and the challenges that bring to reducing energy intake for weight loss. Nevertheless, based on the

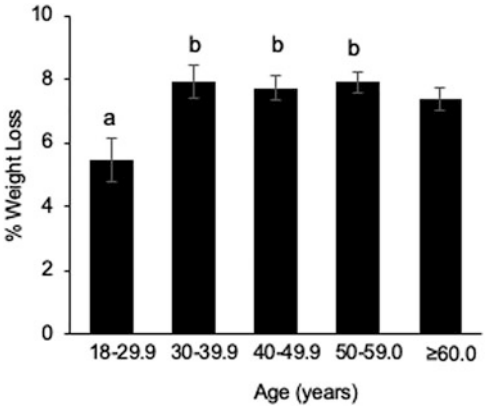

Fig. 3 Association of age with weight loss in a behavioral weight loss intervention (data from Das et al. 2017). Least squares (LS) mean % weight loss ± SE in all participants who completed an 11-week behavioral weight loss intervention ($n = 461$). Values with different superscripts are significantly different, $P < 0.001$ (Das et al. 2017)

physiological changes occurring with aging, dietary advice and intervention design should be adapted to cater to age-specific needs and constraints.

7 Weight Loss Interventions for Underrepresented Groups

In addition to biological and behavioral contributors, obesity as a multi-factorial condition is underlined by social contexts, cultural backgrounds, built environments, and policies (Butland et al. 2007; Mozaffarian 2016). Obesogenic environments are characterized by a disproportionate access, availability, and affordability of energy-dense, nutrient-poor foods, and limited opportunities for physical activity. Populations chronically exposed to environments with these characteristics not only are at greater risk for obesity but may also be more likely to experience the double burden of malnutrition, characterized by both obesity and nutritional deficiencies, particularly in middle-income countries (World Health Organization 2017; Min et al. 2018). When obesogenic environments exist in low-income settings where food insecurity is also prevalent, the lack of reliable and regular access to nutritious foods exacerbates eating practices that promote weight gain (e.g., over-consumption of calories when food is available) which, when combined with chronic stress associated to food insecurity, increase obesity-associated disease risk (Seligman and Schillinger 2010). Therefore, special consideration must be given to the design of weight management interventions that account for the barriers and constraints experienced in such settings.

In the USA, rates of obesity are higher in low-income groups and racial and ethnic minority groups, particularly black and Hispanic/Latino populations with low-income, than in higher income groups and white populations with more resources (Boardman et al. 2005). Emerging research indicates that racial discrimination is an obesogenic risk factor (Hunte and Williams 2009; Hunte 2011; Johnson et al. 2012; Cuevas et al. 2019; Cunningham et al. 2013; Stepanikova et al. 2017). Rates of weight loss in behavioral interventions in these groups are typically low

(Phelan et al. 2017; Perez et al. 2013; McCurley et al. 2017), and evidence suggests that adaptation of weight management strategies to population-specific cultures and backgrounds would increase their effectiveness (Lagisetty et al. 2017). Furthermore, there is a need for investigators and clinicians to proactively target socially disadvantaged populations with higher obesity burden, who tend to be underrepresented in research. A recent systematic review identified several barriers to recruitment of these populations, including lack of awareness about research and health promotion, transportation limitations, and underdeveloped relationships between investigators and communities. Successful recruitment strategies included cultural competence, the formation of robust community-research partnerships, use of media and social marketing, and incentives for research participation (Bonevski et al. 2014).

In addition, an under recognized factor that may contribute to increased risk of obesity in some disadvantaged groups is low energy requirements, as demonstrated by our team and others for Black Americans (Weyer et al. 1999; Sharp et al. 2002; Spaeth et al. 2015; Manini et al. 2011). In carefully controlled studies in a whole-body calorimeter with a standardized exercise regimen and controlling for body mass and composition, Black American women and men have lower sleeping metabolic rate (~100 kcal/day), lower total daily energy expenditure (~140 kcal/day), and higher respiratory quotient (indicating possibly reduced capacity to oxidize dietary fat) compared to White women (Weyer et al. 1999). All of these differences increase the risk that energy intake exceeds energy expenditure (especially in environments where obesogenic diets are the norm) and have been shown to negatively influence behavioral obesity treatment response (DeLany et al. 2014).

8 Summary and Roadmap for Future Personalization Weight Loss Strategies

Weight management interventions frequently lead to a wide range of weight loss and weight regain trajectories among participants, regardless of adherence. The variability in individual response to weight loss may stem from multiple sources, including genetics, metabolic heterogeneity, sociodemographic characteristics, and environments (Berry et al. 2020). Additional factors such as the gut microbiota may also play a role (Fan and Pedersen 2021). This inter-individual variability warrants the design of novel strategies for weight loss (Ordovas et al. 2018). Personalizing interventions, or tailoring to different population groups, is probably the future of weight management interventions but is currently in its infancy (e.g., see (Ebbeling et al. 2007; Pittas and Roberts 2006; Gardner et al. 2018)). In addition to personalizing weight loss approaches to the individual based on metabolism and genetic susceptibility to different diseases that inform dietary recommendations, there is substantial opportunity to personalize dietary recommendations and behavioral counseling based on the individual's lifestyle, age, socioeconomic background, food cultural background, race and ethnicity, and likely other factors. A recently launched International Weight Control Registry (Roberts et al. 2020) may help provide relevant background information on which to test personalized options

versus the minimally tailored current options that are currently in widespread use. The growing obesity crisis worldwide, combined with the demonstrated value of weight loss to combat non-communicable diseases, highlights the great need for progress in improving the effectiveness and sustainability of interventions for routine use. We believe that an important key to the sustainability of interventions is the ability to personalize them to a given individual, since the most effective intervention is likely to be one that they can adhere to over the long-term.

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Physical Activity, Obesity and Weight Loss Maintenance

Claus Brandt and Bente Klarlund Pedersen

Contents

Abstract

Regular physical activity has an impact on all human organ systems and mediates multiple beneficial effects on overall health. Physical activity alone is a poor strategy for weight loss; however, physical activity is of crucial importance for weight loss maintenance. The role of exercise in maintaining a stable body weight is not clear but might be related to better appetite regulation and food preference. In relation to exercise, muscle secretes myokines and other factors that can influence the metabolism in other organs, not least fat and brain tissues. Thereby, physical activity reduces the risk of obesity-associated diseases, such as type 2 diabetes and cardiovascular diseases, independently of weight loss and BMI. Therefore, physical activity should always be included in weight loss strategies

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and as a tool to maintain a healthy weight, despite its modest effect on energy expenditure and overall body weight.

Keywords

Adipose · Cancer · Diabetes · Heart disease · Noncommunicable diseases · Obesity · Physical activity

1 Introduction

In simple terms, accumulation of excessive body fat results from energy imbalance: more calories are consumed than utilized by bodily movement. During the past 30–40 years, energy imbalance has given rise to an increase in the number of obese people (NCD Risk Factor Collaboration (NCD-RisC) 2016). A survey from the Centers for Disease Control and Prevention, conducted between 2013 and 2016, reported that in the last 12 months 49% of the adult American population have attempted to lose weight. The most reported methods were exercising (62.9%) and eating less food (62.9%) (Martin et al. 2018).

Given that physical activity is associated with increased energy expenditure, it is a common belief that physical inactivity leads to obesity, whereas regular physical activity will keep you lean. Moreover, physical activity is the most modifiable component of daily energy expenditure with a potential to create a negative energy balance and thereby reducing body weight. However, in the general population there is little evidence for a causal relationship.

Over the last decades, several studies have attempted to understand the relative contribution by sedentary behaviour and increased caloric intake as contributors to what is often referred to as the obesity pandemic. Understanding the aetiology of the obesity pandemic is obviously of major importance in the prevention of a further increase in the number of obese people. A study from 2009 indicated that increased caloric content of food alone can explain the increase in body weight observed in the USA (Swinburn et al. 2009). However, the number of physically inactive people has increased in parallel and may very well explain at least part of the increase in body weight since the 1970s (Prentice 2007; Millward 2010). Thus, it is still a matter of intense debate to which extent increased caloric intake or decreased physical activity level is responsible for the obesity pandemic. In this context, it is worth noticing that humans have evolved to high levels of daily physical activity and not long periods of sedentary behaviour (Lieberman 2015). In other terms, the bodies of human beings have not yet adapted to the physically inactive lifestyle developed during the past few decades.

Cross-sectional studies show that obese people have lower odds of meeting the physical activity recommendations than people with a normal weight (Churilla et al. 2018), and a recent study identified physical activity level as an important factor in predicting weight status (Cheng et al. 2021). However, a longitudinal study did not support that physical inactivity as reported in a free-living adult population is

associated in the long term with the development of obesity, but the study indicated that obesity may lead to physical inactivity (Petersen et al. 2004). This was supported by a prospective cohort study that included more than 10,000 people from Norway followed over a 16-year period. The study did not find that low levels of neither leisure time nor work-related physical activity would result in weight gain. Instead, supporting the 2004 study, an increase in BMI would result in a decrease in physical activity level (Sagelv et al. 2020, 2021).

Thus, at present, evidence seems to support that an increase in BMI will lead to a decrease in physical activity level, likely because bodily movements become more demanding. Other possible causes may be related to a dopamine dysfunction in the obese state that may further reduce physical activity levels (Kravitz et al. 2016).

This indirectly points to an increased caloric intake being the driver for body weight gain and thereby obesity, leading to an inactive lifestyle. The question remains whether physical activity is (just another) indicator of a healthy lifestyle such as not smoking, eating a healthy diet and getting enough sleep.

In this chapter, we will discuss whether a physically active lifestyle is just a marker of a generally healthy lifestyle, and hence the (potential) role of physical activity for weight management and obesity. We will further discuss whether physical activity has an impact on appetite regulation, food preference, lipid metabolism and behaviour and thereby could serve as an important factor in weight maintenance. In addition, we shall provide evidence that a physically active lifestyle improves health, independently of body mass (Fig. 1).

2 Physical Activity and Weight Loss

Physical activity has the potential to create a negative energy balance and in theory, this holds a great promise for weight loss in overweight or obese humans. However, if a successful weight loss means observing a rapid weight loss on the bathroom scale, exercise alone would have little effect.

The modest success of exercise on body weight reduction is not surprising given the number of calories utilized during the most common exercise programs. Individual sessions are often designed to expend in the range from 500 to 800 kcal per session, commonly performed 3 times per week, corresponding to 1,500–2,000 kcal per week, which theoretically should lead to a monthly weight loss of approximately 1 kg. However, it is suggested that such exercise programs may lead to a compensatory increase in energy intake. Even exercising 5 times per week (400 kcal/session) for 1 year resulted in a total loss of only 3.9 kg fat (Donnelly et al. 2013). Thus, if caloric intake is not decreased, it will be a slow weight loss process and as so little progress is being made, most individuals will fail to obtain a satisfactory body weight loss. Therefore, interventions that solely focus on exercise-induced weight loss with no dietary focus have little clinical relevance in the treatment of obesity. Hence, most studies focus on exercise in combination with diet and behavioural changes.

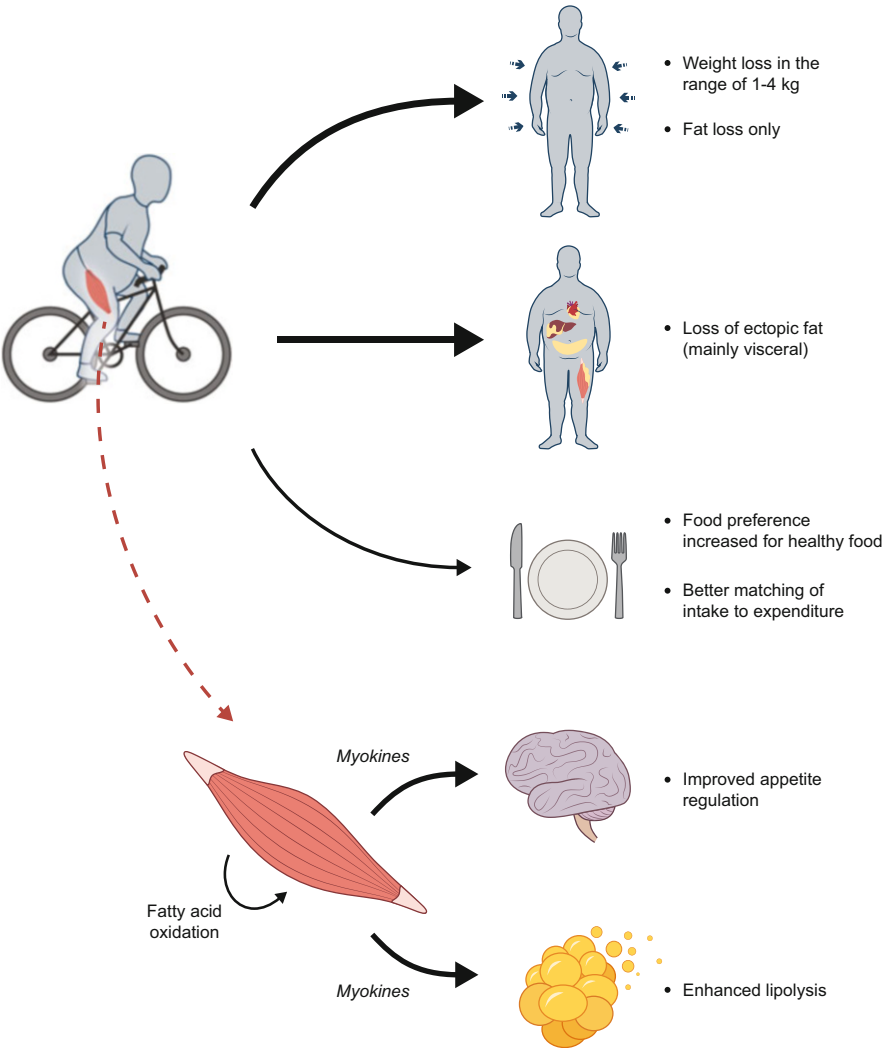

Fig. 1 Effect of physical activity and exercise training on weight loss, fat distribution, food preference, appetite regulation and lipid metabolism

Typically, weight loss programs combining diet, exercise and behavioural changes in overweight and obese subjects aim at reducing body weight by 7–10%, which will improve glycaemia, lipid profile and blood pressure (Wadden et al. 2006) even though the individual will not reach a normal BMI. With such a combinational approach, most of the loss in body weight is derived directly from the reduction in daily caloric intake (Foster-Schubert et al. 2012). Treatment of obesity with the aim of reducing body weight is therefore best achieved by a multifactual approach that

includes behavioural counselling, diet restriction and physical activity in combination (Oppert et al. 2021). Recently, the inclusion of pharmaceutical treatment has increased and a study published in May 2021 showed superiority in body weight reduction when pharmacotherapy was combined with exercise and diet (Marso et al. 2016).

A study comparing the response to a 1-year dietary intervention with a diet + exercise intervention in obese women (>BMI 30) with the aim of losing 10% of their body weight showed that diet on average decreased body weight by 8.5%, exercise alone by 2.4% when performed 5d/week (225 min), and when exercise and diet was combined the average loss reached 10.8% (Foster-Schubert et al. 2012). A systematic review examining all randomized control studies from 1966 to 2008, which included a diet and an exercise + diet group, found that adding exercise to diet increased body weight loss by a further 1.1 kg (Wu et al. 2009). Thus, exercise seems to be additive to the effect of dietary restriction. The small but significant effect of physical activity/exercise training on body weight alone has recently been confirmed in a meta-analysis performed by the European Association for the Study of Obesity physical activity working group – estimating that the addition of exercise would result in losing one additional kg as compared to diet alone (Oppert et al. 2021). Again, this confirms that losing body weight (body fat) from exercise alone is for most people not a good strategy as compared to a reduction in caloric intake when it comes to achieving a meaningful weight loss. However, very strong evidence is provided for the fact that exercise/physical activity has a small but consistent effect on further reducing body weight when combined with diet. This small effect on overall fat mass may "hide" a change in ectopic fat accumulation and overall distribution, which will be discussed later.

When overweight adults engage in an exercise training program with no diet, the expected weight loss would be in the range of 1.5 to 5 kg depending on the amount of exercise performed per week (Bellicha et al. 2021; King et al. 2008; Donnelly et al. 2013). It is notable that when body mass is lost based solely on exercise, only fat mass is lost, whereas a dietary restriction, especially if a very low caloric diet is applied, also results in a loss of lean mass.

An interesting finding from most exercise-only intervention studies is the variability in total fat mass loss. Although fat mass on average is very close to the predicted values from the exercise-induced energy deficit, some individuals lose 3–4 times more and some actually end up gaining weight in the form of 1–2 k of fat (King et al. 2008; Martin et al. 2019; Fearnbach et al. 2020).

Thus, it is important to try to identify those who respond and those who do not in terms of body weight (fat) loss. What are the mechanisms explaining the huge interindividual variation regarding weight loss? At present, data suggest that behavioural responses to food with different caloric content and density differ among those who lose weight and those who do not decrease their body weight (Beaulieu et al. 2020). Humans who lost fat mass after a 12-week exercise training program showed less baseline liking and wanting for high fat foods compared to non-responders (King et al. 2008). This indicates that the response to exercise training relies directly on the behavioural response to food, which depends strongly

on some presently unknown baseline characteristics. The E-MECHANIC trail was designed to address compensatory increase in food intake in response to either moderate amount or a high amount of weekly exercise (Martin et al. 2019) for 24 weeks. As with the 12-week intervention (King et al. 2008), a compensatory increase in energy intake was observed but in a dose-dependent manner with 90% compensating in the high-volume group and 72% in in low volume group. Moreover, the compensatory energy intake was on average higher in the group exercising 5 times per week compared to the 2 times per week group. In the 12-week study, only 13% of the subjects compensated suggesting that compensation takes place when exercise training is repeated regularly over long periods of time. A similar finding has previously been reported (Rosenkilde et al. 2012). Interestingly, here the higher degree of compensation with higher training volume blunted the weight lowering effect so that medium and high-volume training had the same effect on fat mass loss. Again, the subjects were categorized into those who responded with a weight decrease and those who did not lose any weight (Martin et al. 2019). The non-responders differed again at baseline by having a blunted response to low fat food. What is also interesting from this study is that some of the increase in calories may be directly driven by the increase in energy expenditure. The different response in non-responders to high-caloric food is also observed in response to acute exercise, which is in line with the idea (Finlayson et al. 2011) that some baseline feature is responsible for the inability to decrease reward-driven eating behaviour in response to an increase in energy expenditure. In two acute exercise studies, using functional magnetic resonance imaging in healthy young men, a reduced response to high fat food has been shown in reward-related brain regions (Evero et al. 2012; Crabtree et al. 2014). Thus, the normal brain response to exercise may be a reduction in reward-driven caloric intake, which is absent in the non-responders (Beaulieu et al. 2020).

However, more research is needed, probably at the level of the brain, to understand why some subjects do not observe the beneficial effect of exercise on reduction in reward-driven eating in response to exercise training (Hopkins et al. 2014; King et al. 2008). This is likely a barrier that has a large impact on weight loss outcomes in free-living humans trying to lose weight. In a follow-up of the E-MECHANIC, a more careful characterization of the non-responders was performed. Here, the exercise intensity and self-perceived exhaustion during individual training sessions were correlated to less compensation, and thus higher weight loss (Fearnbach et al. 2020).

The finding that non-responders seem to be different from responders already at baseline, i.e. before the intervention, raises some interesting questions (Hopkins et al. 2014; King et al. 2008). Few studies that include exercise consider the relevant question of the individual subjects' life history of physical activity. Being familiar with exercise may result in a different (and better) response to a training intervention (Elsborg et al. 2021). In addition, the trajectory for being overweight in midlife can differ markedly between the individual subjects. Here, it is likely that if exercise used to be part of everyday life, the habituation is faster and thus, behaviour compensation for exercise in terms of self-treatment of unhealthy food is less likely to happen

(Martin et al. 2019). These often-neglected factors in study designs may influence the degree of compensation and hence, weight loss outcome.

In summary, exercise is generally considered an add-on to dietary restriction during weight loss programs. In this setting, exercise blunts the decrease in loss of lean mass and may even preserve lean mass completely, especially if resistance exercise is performed or the caloric restriction is modest. This is a highly desired side effect, particularly in older people, which "counteracts" total body weight loss. Unfortunately, it cannot be measured on a simple scale. Moreover, selective reduction in visceral, pancreatic and hepatic fat content; improvement in physical performance; positive effects on cardiovascular and metabolic health (Battista et al. 2021); and general well-being are also important when considering the beneficial effect of exercise (Carraça et al. 2021). Despite the modest effect of physical activity/exercise on reduction in total body mass, the overall health benefits of exercise go far beyond that of body mass reduction and as such should be strongly recommended for its benefit on overall health independently of changes in body weight.

3 Maintenance of Weight Loss

Weight loss strategies are often successful on a short-term basis. Thus, many people have an initial large weight loss, but often, weight regain occurs rapidly (Purcell et al. 2014; Van Baak and Mariman 2019).

Maintenance of a reduced body weight is associated with compensatory changes in energy expenditure. Weight loss is followed by a reduction in total energy expenditure, beyond that predicted from the weight loss (Leibel et al. 1995). It is generally believed that exercise-induced energy expenditure would close this "gap", which would help maintain a reduced body weight (Melby et al. 2017). It should, however, be noted that the metabolic adaptation to weight loss has recently been questioned. This relates to the time point when energy balance is determined following weight loss (Martins et al. 2020). The more recent finding in larger cohorts suggests that the metabolic adaptation is quantitatively smaller than previously described. If this is true, it would suggest that exercise would become quantitatively more important in maintaining weight loss.

Weight loss is also followed by an increased appetite (Iepsen et al. 2015, 2016) and some (weak) evidence suggests that it may enhance postprandial satiety (Beaulieu et al. 2021).

A classic study by Sumithran et al. (2011) enrolled 50 overweight or obese patients without diabetes in a 10-week weight loss program for which a very-low-energy diet was prescribed. Weight loss was in average 13.5 ± 0.5 kg, which led to reductions in most appetite-regulating hormones, including leptin, peptide YY, cholecystokinin, insulin and amylin and to increases in levels of ghrelin, gastric inhibitory polypeptide and pancreatic polypeptide. There was also a significant increase in subjective appetite. The striking findings were that 1 year after initial weight reduction, levels of the circulating mediators of appetite that encourage weight regain after diet-induced weight loss had not reverted to the levels recorded

before weight loss. In recent years, it has become evident that physical activity/exercise has direct impact on the secretion of hormones from the gastrointestinal tract. An acute bout of exercise decreases acetylated ghrelin and increases both PYY and GLP1 (Schubert et al. 2014). The hormonal profile in the hours following an acute exercise bout resembles that observed following a meal. Presently, it is not known how these changes in GI hormones affect both short- and long-term energy balance. Interestingly, meal-induced suppression of ghrelin and increases in PPY and GLP1 at baseline were linked to a greater weight loss during a 12-week, supervised training program (Gibbons et al. 2017).

Observational studies generally indicate that physical activity has a positive effect on maintenance of weight loss after a diet, as reviewed in Pedersen and Saltin (2015), Dashti et al. (2014). Keeping a reduced body weight requires a fundamental change in lifestyle and whether physical activity is "just" (another) change of habits or if physical activity is necessary to keep a reduced body weight is not known.

The National Weight Control Registry (NWCR) (www.nwcr.ws) was established in 1993 to examine the characteristics of those who are successful at weight loss. The NWCR is tracking over 10,000 individuals who have lost significant amounts of weight and kept it off for at least 1 year. Ninety-eight per cent of the registry participants report that they modified their food intake in some way to lose weight. Ninety-four per cent increased their physical activity, with the most frequently reported form of activity being walking. There is considerable variability in the amount of activity reported: 25.3% report $<$1,000 kcal/week and 34.9% report $>$3,000 kcal/week (Catenacci et al. 2008).

A most recent intervention study (Lundgren et al. 2021) supported the idea that exercise should be included as a means to weight loss maintenance. After an 8-week low-calorie diet with a mean decrease in body weight of 13.1 kg, 195 obese participants were randomly assigned for 1 year to one of four strategies: an exercise program; treatment with liraglutide; exercise program plus liraglutide therapy; or control. At 1 year, all the active-treatment strategies led to greater weight loss than placebo. The combination strategy decreased body fat percentage by 3.9 percentage points, which was approximately twice the decrease in the exercise group and the liraglutide group. As discussed above, most subjects will at some point compensate for the increase in energy expenditure by increasing caloric intake (Westerterp 2018). However, non-responders may compensate earlier and to a greater extent. Given that diet and exercise appear to be additive on weight loss (Bellicha et al. 2021), inhibiting the compensatory increase in food intake with a pharmacological treatment may be an attractive solution in future weight loss strategies. The efficacy of exercise in maintaining weight loss can, however, not be explained by calories spent during exercise alone, suggesting that exercise may influence appetite and/or behaviour.

In summary, regular physical activity may be an important lifestyle intervention in weight loss maintenance. However, it is not known if this is solely due to an effect of exercise on expenditure or if exercise has an impact on appetite or behaviour in a broad sense.

4 Physical Activity and Appetite: Are There an Additional Effect Beyond Burning Calories?

The fact that physically active people have lower-body weight and are leaner (Hall and Kahan 2018) may suggest that exercise has more effects than just burning calories. The first human studies on the relationship between energy expenditure and energy intake date back to 1950s (Mayer et al. 1956). Mayer et al. suggested that at physical activity levels above a certain "threshold", energy intake would be driven more by energy expenditure and in this "regulated zone" a better matching of intake to expenditure would result in a lower and more stable body weight (Mayer et al. 1956). In contrast, in the non-regulated zone below this threshold, energy intake was inversely correlated to energy expenditure. This J-shaped relationship between energy expenditure and energy intake was recently confirmed in a meta-analysis (Beaulieu et al. 2016). Estimations of caloric intake over months to years, relying on self-reported data, are notoriously inaccurate in free-living people (Westerterp 2018). However, to find evidence for such a relationship in humans is difficult as it requires a long study period with strict monitoring of caloric intake. A recent study tried to estimate if higher energy expenditure was associated with better appetite control and decreased risk of overeating, relative to the calories expended during physical activity (Hägele et al. 2019). Although the study was a short-term study conducted in a metabolic chamber within 1 day, it was a randomized, crossover study. At high levels of physical activity, energy intake was better matched to expenditure, which made the authors conclude that "In contrast to the prevailing concept of body weight control, the positive impact of physical activity is independent from burning up more calories and is explained by improved appetite sensations" (Hägele et al. 2019). This follows the theory of Mayer (Mayer et al. 1956) that homeostatic mechanisms match intake to expenditure only when expenditure is moderate to high. Such a homeostatic regulation, saying that intake drives expenditure, still needs to be validated outside the metabolic chamber over longer time periods. Other short-term approaches have suggested that physically active individuals have a better appetite control. Beaulieu and Long tested energy compensation in humans with low, medium and high levels of self-reported physical activity (Beaulieu et al. 2017; Long et al. 2002). Integration of calories given orally before an ad libitum meal was improved in individuals with medium and high levels of physical activity. However, the improved integration was only observed when the number of calories was relatively high.

In summary, even if high levels of weekly energy expenditure lead to a better matching of energy intake to expenditure through enhanced sensitivity in some unidentified homeostatic systems, this mechanism is easily overridden by "human behaviour". In order to understand why and how the behavioural responses related to caloric intake at the individual level are so variable in response to exercise training in obese individuals, it will be important to understand why some people gain weight after an exercise intervention, despite completing 95% of the training sessions. Here, sophisticated brain scans may be an efficient tool to highlight differences in brain activity that may or may not provide support for physical activity/exercise to change

behavioural responses towards high fat foods, and ultimately such a strategy may help in controlling body weight. However, at present, the direct evidence for a strong effect of exercise on appetite control is still weak (Beaulieu et al. 2021).

5 Physical Activity, Fitness and Fat Distribution

As discussed above, loss of fat mass is often small in response to an increase in physical activity. However, even if fat mass as such is not lost, visceral fat mass can be reduced by increasing physical activity, suggesting that physical activity leads to a redistribution of fat between different fat compartments. Visceral fat mass is conversely related to cardio-metabolic health and the selective effect of physical activity on this compartment is important, even in the absence of total fat loss.

Using BMI alone in predicting metabolic health is controversial (Boonchaya-Anant and Apovian 2014; Kramer et al. 2013; Oliveros et al. 2014). Studies have indicated that individuals can be obese and metabolically healthy (high insulin sensitivity, low abdominal adiposity and low levels of inflammation) or they can be of normal weight but with an unhealthy metabolic profile (Boonchaya-Anant and Apovian 2014; Oliveros et al. 2014; Wajchenberg 2000). The metabolically healthy, obese phenotype represents up to 30% of obese individuals (Boonchaya-Anant and Apovian 2014).

Abdominal adiposity and low fitness are both associated with cardiovascular diseases, type 2 diabetes, dementia, cancer (Pedersen 2009) and all-cause mortality, independently of BMI (Pischon et al. 2008). Thus, the consequences of abdominal adiposity and low fitness are overlapping. Abdominal adiposity reflects the amount of visceral fat mass (Wajchenberg 2000), which is more characterized by being more inflamed than subcutaneous fat (Ibrahim 2010).

In an epidemiological, cross-sectional study of 10,976 free-living people, an inverse association between fitness level and waist circumference and an inverse association between fitness and high sensitivity CRP were found within all BMI categories. Thus, a low fitness level is associated with both abdominal adiposity and low-grade inflammation, independently of BMI. These cross-sectional data suggest that, despite BMI, an increase in fitness level may lead to a reduction in abdominal fat mass and low-grade inflammation. This hypothesis is confirmed by intervention studies that provide strong evidence that physical training effectively reduces the amount of visceral adipose tissue (Maillard et al. 2018; Sabag et al. 2017) and lowers chronic inflammation (Pedersen 2017; Karstoft and Pedersen 2016a).

The idea that a low fitness and/or physical activity levels lead to accumulation of visceral fat has also been supported by intervention studies. A direct link between physical inactivity and visceral fat has been established in both rodents (Laye et al. 2007) and humans (Olsen et al. 2008; Krogh-Madsen et al. 2014).

More than a decade ago, we developed a real-life, physical inactivity model in which healthy young volunteers declined their number of daily steps. In the first study, a group of young healthy men decreased their daily stepping for 14 days to 1,500 steps from the range recommended for adults of around 10,000. During this

time, they developed a markedly impaired glucose tolerance as well as attenuation of postprandial lipid metabolism. The intervention was also associated with a 7% increase in intra-abdominal fat mass, measured by MR-scanning, without a change in total fat mass, while total fat-free mass and body weight decreased (Olsen et al. 2008). A follow-up study revealed that the volunteers developed a marked decline in peripheral insulin sensitivity without an effect on hepatic endogenous glucose production. In addition, the 2-week period induced a 7% decline in VO_2max (ml/min; cardiovascular fitness) (Krogh-Madsen et al. 2010). Thus, the consequence of physical inactivity is accumulation of visceral fat mass and impaired peripheral insulin sensitivity.

Another study included healthy individuals on a high-caloric food intake who were randomized to either 10,000 or 1,500 steps/day for 14 days. Both study groups received a diet compounded to secure that they gained the same amount of body weight. However, the inactive group accumulated significantly more visceral fat compared to the active group. Following the 2-week period, the inactive group also experienced a poorer glycaemic control, increased endogenous glucose production, decreased hepatic insulin extraction, increased baseline plasma levels of total cholesterol and LDL and a decreased cognitive function with regard to capacity of attention (Krogh-Madsen et al. 2014). This study supports that habitual physical activity may prevent pathophysiological symptoms associated with diet-induced obesity.

Thus, a physically inactive lifestyle and poor fitness lead to accumulation of visceral fat, whereas regular exercise training reduces abdominal adiposity and inflammation. It has been suggested that subcutaneous adipose tissue, particularly in lower-body fat depots, may protect against chronic diseases. In contrast, strong evidence exists to the detrimental effects of the accumulation of visceral fat, and this latter depot as well as fat in the liver and in skeletal muscle (Pischon et al. 2008) may stimulate an inflammatory response (Yudkin 2007). We have suggested that the accumulation of visceral fat will activate, over time, a network of inflammatory pathways that promote the development of insulin resistance, atherosclerosis, malignancy and neurodegeneration, as well as a network of chronic diseases, including cardiovascular diseases, type 2 diabetes, cancer and Alzheimer's disease (Benatti and Pedersen 2015).

In summary, strong evidence exists that even short-term physical inactivity will lead to an accumulation of visceral fat, whereas physical training decreases the amount of visceral fat mass, independently of total fat mass and BMI.

6 Myokines: Effect on Adipose Tissue and Brain

Skeletal muscle cells are highly metabolically active, and during exercise, skeletal muscles communicate with other organs by producing and releasing the so-called myokines. The skeletal muscle secretome in humans consists of hundreds of myokines that are secreted from muscle cells during proliferation and differentiation or in response to muscle contractions. Myokines can exert autocrine, paracrine or

endocrine effects (Giudice and Taylor 2017; Karstoft and Pedersen 2016b; Pedersen 2013; Pedersen and Febbraio 2012; Hoffmann and Weigert 2017; Schnyder and Handschin 2015; Raschke and Eckel 2013; Pedersen 2019; Severinsen and Pedersen 2020).

Some myokines are involved in energy supply during acute exercise, and repetitive acute bouts are probably involved in mediating adaptation to training in various organs. Myokines mediate signalling within the muscle itself and muscle–organ crosstalk to the liver, gut, pancreas, adipose tissue, bone, vascular bed and skin (Severinsen and Pedersen 2020). Of interest to the theme of this chapter is the role of myokines in mediating some of the effects of exercise on adipose tissue, brain and muscle.

6.1 Muscle–Adipose Crosstalk

A world of literature has proven that IL-6 is released from contracting human muscle cells into the circulation and that it contributes to the exponential increase in plasma-IL-6 in relation to exercise, reviewed in Pedersen (2013, 2017); Knudsen and Pedersen (2015); Karstoft and Pedersen (2016a, b); Benatti and Pedersen (2015). The effect of exercise-induced IL-6 on fat metabolism is one of the most well-supported findings (Pedersen 2013, 2018). In vitro studies and studies in rodents show that IL-6 can enhance lipolysis and fat oxidation, via a mechanism that involves AMP-activated protein kinase activation (Pedersen and Febbraio 2008). In vivo studies show that rhIL-6 enhances lipolysis and fat oxidation in healthy young and elderly humans (Petersen et al. 2005; Van Hall et al. 2003; Wolsk et al. 2010).

As pointed out previously, exercise training reduces abdominal adiposity via a mechanism that includes IL-6 (Wedell-Neergaard et al. 2019). Abdominally obese adults were randomized to tocilizumab (IL-6 receptor antibody) or placebo during an intervention of 12 weeks with either aerobic exercise or no exercise (Wedell-Neergaard et al. 2019; Christensen et al. 2018). As expected, exercise training led to a reduction in visceral adipose tissue mass. However, this effect was abolished by the IL-6 receptor blockade (Wedell-Neergaard et al. 2019). Moreover, IL-6 receptor blockade abolished the exercise-induced loss of cardiac fat (Christensen et al. 2019).

Brown fat expresses a set of proteins, such as uncoupling protein 1 (UCP1). White adipose tissue can shift into a brown-like phenotype, and thus, the discovery of brown fat in humans and the potentially beneficial effects of these depots have stimulated a number of studies to explore whether lifestyle, such as exercise, can contribute to induce browning of white fat (Townsend and Wright 2019; Rodriguez et al. 2017; Eckel 2019).

In 2012, irisin was reported as a myokine with the ability to brown white adipose tissue in mice (Bostrom et al. 2012). However, while evidence exists that irisin is released from rodent muscle and has browning effects, it is debated if exercise leads to an increase in plasma-irisin levels in humans (Albrecht et al. 2015; Dinas et al. 2017).

A couple of other exercise-induced myokines with browning effects have been identified. In 2014, Spiegelman's group identified meteorin-like (Metrnl), a circulating muscle-derived factor, that is induced in muscle after exercise. Metrnl stimulates the expression of genes associated with beige fat thermogenesis. Further, it stimulates energy expenditure and improves glucose tolerance. Yet, the role of Metrnl in humans remains to be identified.

Studies suggest that IL-6 can induce browning of white adipose tissue. Daily intraperitoneal injections of IL-6 to mice for 1 week increased *ucp1* mRNA in inguinal white adipose tissue (iWAT) (Knudsen et al. 2014). Moreover the authors used whole-body IL-6 KO mice and demonstrated that 5 weeks of endurance training increased ucp1 mRNA only in wild type mice and not in mice lacking IL-6 (Knudsen et al. 2014). In a follow-up study the effect on ucp1 mRNA was recapitulated in mice lacking IL-6 only in muscle (Knudsen et al. 2017). A study by Kristof et al. (2019) found that IL-6 was mainly produced by fully differentiated adipocytes. When the IL-6 receptor was blocked during differentiation, brown marker genes were downregulated, suggesting that beige adipocytes regulate IL-6 production to enhance browning in an autocrine manner. It remains to be shown that the physiological concentrations of IL-6, released during exercise, have browning effects.

There are a few other circulating factors during exercise, which have the potential to induce browning. β-aminoisobutyric acid (BAIBA) is a small molecule, a non-protein beta-amino acid, not classified as a myokine, but secreted from myocytes (Roberts et al. 2014; Kammoun and Febbraio 2014). Moreover, BAIBA has browning effects on human adipocytes (Roberts et al. 2014; Kammoun and Febbraio 2014). In addition, two hepatokines appear to play a role in exercise-induced browning of white adipose tissue. The fibroblast growth factor 21 (FGF21) (Hansen et al. 2015) and Follistatin (Hansen et al. 2016b) are released from human liver during exercise and this release is controlled by the glucagon-to-insulin ratio (Hansen et al. 2016a). Evidence exists that both Follistatin (Singh et al. 2014) and FGF21 (Veniant et al. 2015) can induce browning of white adipose tissue cells. However, it should be noted that the physiological effect of physical activity on adipose tissue browning is presently only demonstrated in rodents.

6.2 Muscle–Brain Crosstalk

Evidence is accumulating that physical exercise has positive effects on cognitive function and brain health (Cotman et al. 2007; Mattson 2012). Moreover, physical activity has beneficial effects on sleep (Kelley and Kelley 2017) and mood (Crush et al. 2018).

As said, during muscle work, IL-6 is produced by contracting muscle cells and released into the blood (Febbraio and Pedersen 2002) in a TNF-independent fashion (Keller et al. 2006). The release of IL-6 leads to an exponential rise in circulating concentrations of IL-6. Systemic IL-6 KO mice accumulate adipose tissue (Wallenius et al. 2002; Matthews et al. 2010), whereas central overexpression of

IL-6 (Hidalgo et al. 2010; Senaris et al. 2011) leads to a decrease in body weight, indicating that IL-6 is a player in body weight control. Another murine study demonstrated that lack of muscular IL-6 led to a decrease in body weight and food consumption in response to leptin (Molinero et al. 2017).

A study showed that IL-6 improves glucose tolerance and suppresses feeding, when it is applied centrally in mice, but not intraperitoneally at the same dose (Timper et al. 2017). However, a four-fold higher IL-6 concentration injected peripherally significantly reduced food intake. This finding suggests that high systemic concentrations of IL-6 can pass the blood-brain barrier and exert central effects on appetite. Thus, it is likely that muscle-derived IL-6, elicited by exercise of long duration and high intensity, may inhibit appetite. Inactivation of the *IL-6* gene only in skeletal muscle of mice altered the expression of *pomc, agrp and npy,* which are key hypothalamic neuropeptides in the control of not only food intake but also whole-body energy balance. The effect was gender specific, with females having an increased expression of *npy* and *agrp,* reduced *pomc* expression and increased body weight. These data pointed towards muscle secreted IL-6 directly controlling energy balance at the level of the hypothalamus (Ferrer et al. 2014).

6.3 Muscle–Muscle Crosstalk

Myostatin is the first identified muscle-derived factor that fulfils the myokine criteria as outlined above (Mcpherron et al. 1997). Myostatin is a member of the transforming growth factor b superfamily and negatively regulates myogenesis in an autocrine manner (Mcpherron et al. 1997). Massive muscle hypertrophy is seen in myostatin KO mice, cattle, sheep and dogs (Mcpherron et al. 1997; Mosher et al. 2007; Grobet et al. 1997) that demonstrate an increase in fibre cross-sectional area as well as in fibre number. Myostatin mice are not only protected from diet-induced obesity (Mcpherron and Lee 2002), but in response to an increase in caloric density, the mice are better in matching energy intake to energy expenditure (Bond et al. 2016), highlighting muscle mass as a regulator of energy balance.

Decorin has been identified as a myokine that is regulated by exercise and acts as an antagonist to myostatin (Kanzleiter et al. 2014). Circulating levels of decorin are increased in response to exercise in humans (Kanzleiter et al. 2014), whereas exercise training reduces the levels of myostatin within muscles and blood (Saremi et al. 2010; Hittel et al. 2010).

Although the myokine IL-6 is mostly recognized for its regulatory effects in lipid and glucose metabolism, IL-6 is also playing important roles in myogenesis. Muñoz-Cánoves and her team identified IL-6 as an anabolic factor. Using a model of compensatory hypertrophy, genetic loss of IL-6 impaired muscle hypertrophy in vivo, whereas myotube-produced IL-6 stimulated muscle cell proliferation in a paracrine fashion (Serrano et al. 2008).

Leukaemia inhibitory factor (LIF) is a member of the IL-6 cytokine superfamily and has multiple biological functions. LIF protein has been shown to be secreted from human cultured myotubes, when electrically stimulated (Broholm et al. 2008)

and LIF stimulates satellite cell proliferation (Broholm and Pedersen 2010). It has further been shown that both IL-6 and LIF activate myotube mTORC1 signalling in a time- and dose-dependent fashion (Gao et al. 2017). A number of other myokines, including IL-15 (Nielsen et al. 2007) and IL-7 (Haugen et al. 2010), have further been demonstrated to possess anabolic features.

In summary, in relation to exercise, muscle secretes myokines and other factors that may influence the metabolism in other organs, not least fat tissue and brain tissue, which may contribute to mediate the health beneficial effects of physical activity and exercise training.

7 Conclusion

Regular, physical activity has an impact on all human organ systems and results in a plethora of beneficial effects on overall health. Physical activity alone is a poor strategy for weight loss. However, physical activity adds to the effect of diet and appears to be of crucial importance for weight loss maintenance. Moreover, physical activity decreases the risk of obesity-associated diseases, independently of weight loss and BMI. Therefore, physical activity should always be included in weight loss strategies and as a tool to maintain a healthy weight, despite its modest effect on overall body weight.

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Bariatric/Metabolic Surgery

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Abstract

Bariatric surgery is a gastro-intestinal surgery aimed at obtaining weight loss in obesity. The rapid metabolic effects of this type of operations provided a rational to change its name to metabolic surgery, in fact often the improvement of metabolic diseases is observed before a meaningful weight reduction.

In this review, we examine the effects of laparoscopic metabolic surgery on life expectancy, type 2 diabetes, hypertension, cardiovascular disease and cancer.

Furthermore, we review the surgical endoscopy approaches to obesity including primary obesity procedures and revision procedures that address weight regain after bariatric surgery.

Finally, as a bridge to the specific chapter, we summarize the effects on weight reduction of new anti-obesity medications.

Keywords

Bariatric surgery · Endoscopy · Laparoscopy · Metabolic surgery · Minimally invasive surgery

Gastrointestinal surgical weight loss procedures, also called bariatric surgery after the ancient Greek words "baros", meaning weight, and "iatros", meaning cure, include laparoscopic and minimally invasive endoscopic surgical approaches.

Differently from lifestyle modification and anti-obesity medications, effectiveness and durability are two important attributes of bariatric surgery.

The weight loss achieved after bariatric surgery depends on the type of operation performed; Roux-en-Y Gastric Bypass (RYGB) is associated with approximately 20% total weight loss (%TWL), Sleeve Gastrectomy (SG) with 15% and gastric banding with 10% at 1-year follow-up (Seo et al. 2016). The higher the baseline weight, the higher the reduction.

Weight loss after bariatric surgery follows a normal distribution (Pucci and Batterham 2019) with some people losing up to 60% of their baseline weight and others only 5% and the great majority lying in the middle.

Insufficient weight loss, defined as <50% excess weight loss (EWL), is the most common reason to qualify for revisional bariatric surgery. Some weight regain is common, however 20–25% of patients fight with a considerable weight regain. A weight regain of 38% of the maximal weight loss obtained at 1 year after surgery was observed after laparoscopic adjustable gastric banding (LAGB) (Sjöström et al. 2004), while it was approximately 27.8% after SG (Clapp et al. 2018) and 4% after RYGB (Courcoulas et al. 2018). However, these figures derive from relatively small studies and it is difficult to generalize to the entire population of subjects operated of bariatric surgery.

Some of the reasons for weight regain are the enlargement of the stoma size, the dilation of the gastric pouch or in some cases the occurrence of a gastro-gastric fistula, but also genetic predisposition to obesity or hormonal or metabolic causes

can intervene. For instance, reactive hypoglycaemia (Castagneto-Gissey and Mingrone 2012) that can occur after RYGB or SG increases the need to eat sugar rich meals with consequent weight gain. Depression or binge eating disorders can induce the patient to overeat as a psychological compensatory mechanism. In these cases, pharmacological treatment of depression associated with behavioural therapy can avoid a new surgical operation.

Anti-obesity medications such as phentermine and phentermine–topiramate treatment for 3 months were associated with 6.35 kg and 3.81 kg weight loss, respectively, in patients who regained weight after RYGB (Schwartz et al. 2016). Also liraglutide, a GLP1 receptor agonist induced a 6.3 ± 7.7 kg weight loss after 7 months of therapy after either RYGB or SG (Wharton et al. 2019). The surgical revision of RYGB for weight regain, with conversion to distal RYGB, BPD/DS or re-sizing of the gastric pouch and anastomosis, placement of a gastric band or by endoscopic procedures, allowed a reduction of the excess body mass index of 52.2%, 76%, 14%, 47.3% and 32.1%, respectively, after 3 years (Tran et al. 2016).

The effects of gastro-intestinal surgery on obesity-related comorbidities are observed just few days after the operation, particularly after BPD with or without duodenal switch, when the body weight is almost unchanged. Hence, the name was changed from bariatric to metabolic surgery to highlight the fact that also metabolic improvement, along with weight loss, is one of the main effects of surgery.

1 Laparoscopic Bariatric/Metabolic Surgery

There are several types of bariatric procedures performed laparoscopically. Sleeve Gastrectomy (SG), Roux-en-Y Gastric Bypass (RYGB), Biliopancreatic diversion with Duodenal Switch (BPD-DS) and Single Anastomosis Duodeno-Ileal bypass (SADI-S) are the most common.

1.1 Sleeve Gastrectomy

SG was initially envisioned as the first step of a more complex operation, the BPD-DS. The procedure was performed in two stages especially in high risk patients in order to reduce perioperative complications. Thanks to its effectiveness and safety profile, SG gained widespread popularity, becoming the most performed operation first in the USA and then in Europe.

It encompasses the tubulization of the stomach along its lesser curvature (Fig. 1a). The greater curvature is skeletonized and a stapler is then used along a 36–50 F. bougie, starting 4–6 cm from the pylorus and continuing up to the angle of His. A gastric “sleeve” with a capacity of 60–100 ml.

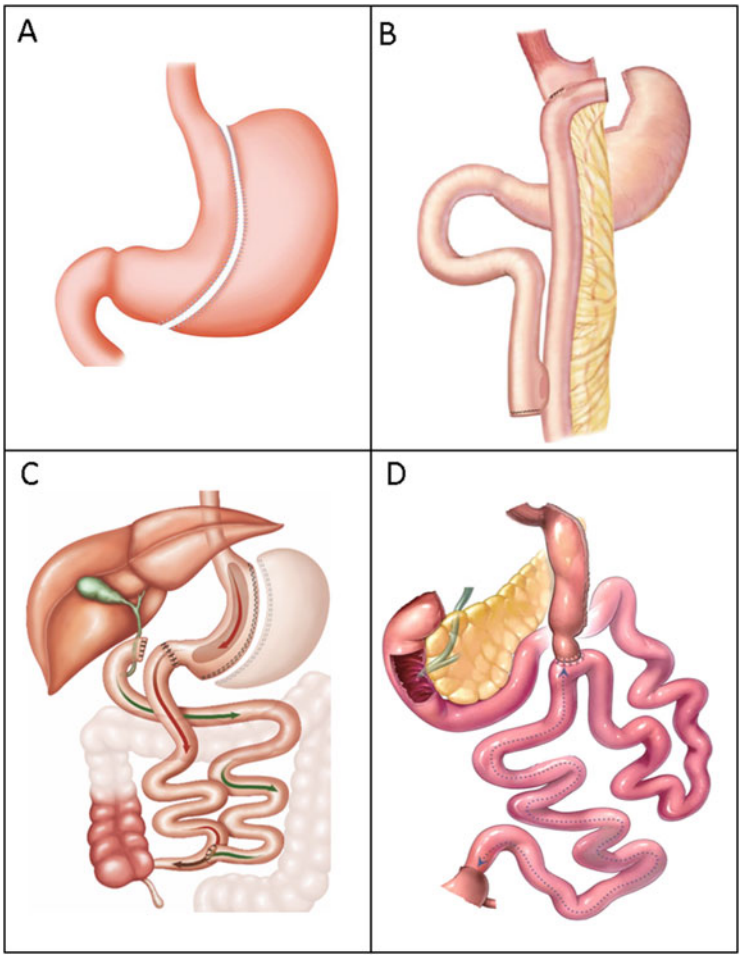

Fig. 1 Bariatric/metabolic surgical procedures (**a**) Sleeve gastrectomy, (**b**) Roux-en-Y gastric bypass, (**c**) Biliopancreatic diversion with duodenal switch, (**d**) Single anastomosis duodeno-ileal bypass

1.2 Roux-en-Y Gastric Bypass

Originally the gold standard bariatric operation, RYGB is now the second most executed operation worldwide. A gastric pouch of approximately 30 ml is made. The bowel is measured and divided approximately 75 cm from the Treitz ligament which will represent the biliopancreatic limb. A gastro-jejunostomy is performed and a second anastomosis is made approximately 100 cm caudad, representing the alimentary limb (Fig. 1b).

1.3 Biliopancreatic Diversion With or Without Duodenal Switch

BPD-DS represents a modification of the original biliopancreatic diversion developed by Scopinaro. BPD-DS can be performed as a one- or two-staged operation. The presence of severe comorbidities or super-obesity (BMI > 50 kg/m^2) is often an indication to a staged procedure in order to reduce perioperative risks. An SG is first completed and then divided 1–2 cm below the pylorus. Duodeno-ileal and ileo-ileal anastomoses are performed, leaving an alimentary limb of 250 cm and a common channel of 150 cm (Fig. 1c).

1.4 Single Anastomosis Duodeno-Ileal Bypass

SADI-S was initially thought as an operation for inadequate weight loss or weight regain after SG, but over time became a standalone operation. SADI-S (Fig. 1d) consists of a sleeve gastrectomy and of an end-to-side anastomosis of the first part of the duodenum, 2–4 cm distal to the pyloric ring, with 250–300-cm proximal ileum to ileocecal junction. Contrary to BPD-DS, SADI-S avoids the need for a jejuno-ileal anastomosis reducing the time and the complexity of the operation.

Long-term data are largely lacking due to its recent introduction as a bariatric procedure. Weight loss at 1 year after surgery ranges from 21.5% to 41.2% and from 25.8 to 46.3% at 2 years (Spinos et al. 2021). Early (<30 days) postoperative complications are between 5.3% and 0.5% and include reoperations (3.1%), bleeding (1.1%), wound infection (1.0%), anastomotic leak (0.9%) and intra-abdominal abscess (0.6%) (Spinos et al. 2020). Long-term nutritional deficiencies include hypoproteinaemia, as well as vitamin and micronutrient deficiencies (Balibrea et al. 2017).

1.5 Effects on Life Expectancy

Bariatric/metabolic surgery is associated with an overall reduction in mortality hazard rate of 49.2% (95% CI 46.3–51.9, $P < 0.0001$) and increases median life expectancy of 6.1 years (95% CI 5.2–6.9) as compared with standard of care. This effect is more pronounced in subjects with type 2 diabetes, who have an 82% higher effect with a median life expectancy of 9.3 years (95% CI 7.1–11.8) compared with non-diabetic individuals who have a life expectancy gain of 5.1 years (95% CI 2.0–9.3). No significant difference was observed between RYGB and SG (I^2 3.4%, $P = 0.36$). The above meta-analysis estimated that every 1% increase in bariatric/metabolic surgery utilization would have an incredibly large benefit in terms of life expectancy that would increase by 5.1 million and 6.6 million worldwide for patients with or without diabetes, respectively (Syn et al. 2021).

1.6 Effects on Type 2 Diabetes

Bariatric/metabolic surgery determines type 2 diabetes remission, avoiding thus the utilization of anti-diabetic medications.

In a randomized controlled trial with 2 years' follow-up (Mingrone and Castagneto-Gissey 2014), diabetes remission – defined as a glycated haemoglobin lower than the threshold of diabetes, i.e. <6.5%, in association with fasting plasma glucose <100 mg/dl – occurs in 75% of subjects who underwent RYGB and in 95% of those who underwent BPD, while no diabetes remission was present in patients under medical treatment alone ($P < 0.001$ for both comparisons). Starting from a baseline value of HbA1c of 8.65 ± 1.45%, at 2 years after surgery it decreased to 7.69 ± 0.57% in the medical-therapy group, 6.35 ± 1.42% in the RYGB group, and 4.95 ± 0.49% in the BPD group. Therefore, BPD shows the most striking effects that persist a long time after surgery. At 5-year follow-up, diabetes remission was still present in 50% of subjects with RYGB and in 63% in those with BPD (Mingrone et al. 2015). At 10-year follow-up, 25% of subjects who underwent RYGB and 50% of subjects who had BPD were under diabetes remission (Mingrone et al. 2021a).

In the study of Schauer et al. (2012), the proportion of patients reaching HbA1c ≤6% with or without anti-diabetic medications was 12% in the medical-therapy group, 42% in the RYGB group ($P = 0.002$) and 37% in the SG group ($P = 0.008$). The mean HbA1c was 7.5 ± 1.8% in the medical-therapy group, 6.4 ± 0.9% in the RYGB group ($P < 0.001$) and 6.6 ± 1.0% in the SG group ($P = 0.003$). Weight loss at 1 year after bariatric/metabolic surgery was similar after RYGB (−29.4 ± 9.0 kg) and SG (−25.1 ± 8.5 kg), almost 5 times greater compared to the medically-treated group (−5.4 ± 8.0 kg) ($P < 0.001$ for both comparisons).

At 5-year follow-up, only 5% of patients who received medical therapy alone had a HbA1c ≤6%, while 29% of those who underwent RYGB (unadjusted $P = 0.01$, adjusted $P = 0.03$, $P = 0.08$ in the intention-to-treat analysis) and 23% of those who underwent SG (unadjusted $P = 0.03$, adjusted $P = 0.07$, $P = 0.17$ in the intention-to-treat analysis) met the primary end-point of HbA1c ≤6% (Schauer et al. 2017).

A large monocentric study showed a type 2 diabetes remission rate of 64.7%, while 23.5% had a substantial improvement of glycaemic control at 10 years after SG (Castagneto Gissey et al. 2018a). Similarly, the Swiss Multicentre Bypass or Sleeve Study, a randomized controlled trial, found a diabetes resolution rate of 61.5% which was superimposable to RYGB (67.9%) at 5 years postoperatively (Peterli et al. 2013).

1.7 Effects on Hypertension and Cardiovascular Diseases

The Swedish Obese Subjects (SOS) study is a prospective intervention study specifically designed to investigate the effect of weight loss on mortality and other obesity-related comorbidities. In this study, the patients underwent different types of bariatric operations or lifestyle modification interventions.

In a recent publication with 20-year follow-up (Carlsson et al. 2020), the hazard ratios for the bariatric surgical group as compared with the control group were 0.70 (95% CI, 0.57–0.85) for any cardiovascular disease, 0.51 (95% CI, 0.33–0.79) for myocardial infarction, 0.52 (95% CI, 0.31–0.88) for heart failure and 0.45 (95% CI, 0.24–0.84) for stroke. Also the mortality due to cancer was significantly reduced after bariatric surgery, with a hazard ratio of 0.77 (95% CI, 0.61–0.9).

In another study, bariatric/metabolic surgery was associated with a 33% (HR, 0.67 [95% CI, 0.52–0.87]) and 60% (HR, 0.40 [95% CI, 0.25–0.63]) lower hazard of a second ischemic event or worsening of heart failure, respectively (Doumouras et al. 2021).

RYGB improves hypertension management with 30% or more reduction of antihypertensive medications occurring in 73% of patients from the RYGB group compared with 11% of patients from the medical-therapy group (relative risk, 6.52 [95% CI, 2.50–17.03]; $P < 0.001$) (Schiavon et al. 2020).

1.8 Effects on Cancer

It is well known that obesity increases the risk of cancer. In a retrospective study comparing the incidence of cancer among 6,596 patients who had RYGB between 1984 and 2002 and 9,442 people with severe obesity along 24-year timeframe, the incidence of cancer was 24% lower in the group of patients who had undergone RYGB (hazard ratio (HR), 0.76; 95% CI, 0.65–0.89; $P = 0.0006$). However, this effect was present only in women (HR, 0.73; 95% CI, 0.62–0.87; $P = 0.0004$), but not in men (HR, 1.02; 95% CI, 0.69–1.52; $P = 0.91$). In particular, a significant decrease in uterine cancer incidence was observed (HR, 0.22; 95% CI, 0.13–0.40; $P < 0.0001$) (Adams et al. 2009).

In the SOS study with 10-year follow-up, the incidence of malignancies was significantly lower in the surgical group ($n = 117$) than in the control one ($n = 169$; HR 0.67, 95% CI 0.53–0.85, $P = 0.0009$). This study confirmed that it was the outcomes in the female group who had driven the significant results with HR of 0.58 (0.44–0.77; $P = 0.0001$), whereas there was no effect of surgery in men (Sjöström et al. 2009).

On the other hand, some authors have reported an increase in some types of cancers. Cases of gastric cancer of the excluded stomach have been described after RYGB. Biliary and pancreatic juices can accumulate in the gastric remnant and this has been hypothesized to cause a progression from intestinal metaplasia to adenocarcinoma (Castagneto-Gissey et al. 2020). Several studies showed how SG is associated with an elevated risk of developing Barrett's oesophagus, with an incidence of approximately 8% (Yeung et al. 2020). Barrett's oesophagus is by all means a precancerous lesion and can progress to oesophageal adenocarcinoma. A recent literature review described 7 cases of oesophageal adenocarcinoma after SG, estimating how this procedure could raise the risk of developing oesophageal cancer by 110 new cases every 3 years (Genco et al. 2021).

2 Endoscopic Bariatric Surgery

Surgical endoscopy for obesity includes primary obesity procedures and revision procedures addressing weight regain after bariatric surgery. The way through which it works is the reduction of food intake either by reducing the gastric volume or by decreasing food content through aspiration. Another approach is to increase gastric emptying via gastric electrical stimulation or vagal nerve blocking.

2.1 Intragastric Balloons

Intragastric balloons are positioned into the stomach under light sedation and are then filled with 400–700 ml of saline solution containing methylene blue for early detection of balloon perforation. They are left in place for up to 6 months. A plethora of gastric balloons exists, such as BioEnterics Intragastric Balloon (BIB; Allergan Inc., Irvine, CA, USA), the Spatz adjustable balloon system (Spatz FGIA, Inc., Great Neck, NY, USA) and ReShape dual intragastric balloon system (ReShape Medical, San Clemente, CA, USA) among others.

The ReShape Integrated Dual Balloon System consists of two balloons connected in the middle. It was created to conform to the natural shape of your stomach and to reduce the discomfort and nausea that can accompany intragastric balloon treatment. However, nausea and vomiting were 87% and 61%, respectively (https://www.accessdata.fda.gov/cdrh_docs/pdf14/P140012d.pdf).

Other balloons, such as Obalon gastric balloon (Obalon Therapeutics Inc., Carlsbad, CA, USA), Heliosphere BAG balloon (Helioscopie Medical Implants, Vienne, France) and Ullorex oral intragastric balloon (Phagia Technologies, Inc., Fort Lauderdale, FL, USA), are instead inflated with gas. Their size is smaller than that of fluid-filled balloons and so is the effectiveness in reducing body weight. The Obalon gastric balloon has the advantage that it is contained in a swallowable capsule made of gelatin that dissolves in the gastric juice and is inflated from outside with gas via a micro-catheter to a maximal size of 250 ml, 1–3 balloons are usually necessary to obtain sufficient weight loss.

All the above balloons are removed endoscopically under sedation.

The Ullorex OIB is a large capsule containing $NaHCO_3$, which is injected with citric acid and swallowed. After few minutes it expands to 300 ml thanks to the formation of CO_2. After 25–30 days, its wall degrades in the acid milieu of the stomach permitting the expulsion of the balloon with the faeces. However, after a first study including 12 subjects (Martin et al. 2007) in which the median weight loss was 6.5 kg, no further studies were published.

2.2 Hydrogel

Gelesis100 is a superabsorbent hydrogel made of cellulose cross-linked with citric acid and content in capsules that once swallowed dissolve in the stomach forming

small gel aggregates occupying about one fourth of the gastric volume. Gelesis100 induced a significantly ($P = 0.0007$) higher weight loss of $-6.4 \pm 5.8\%$ than placebo ($-4.4 \pm 5.5\%$). Fifty-nine of the patients treated with Gelesis100 lost $\geq 5\%$ weight loss compared with 42% in the placebo group and 27% vs. 15% had a weight loss $\geq 10\%$ (Greenway et al. 2019). Side effects were minor and gastro-intestinal in nature.

2.3 Endoluminal Vertical Gastroplasty

Different endoscopic devices are available for endoluminal vertical gastroplasty. The EndoCinch Suturing System (C.R. Bard, Murray Hill, NJ, USA) was initially developed to treat the gastroesophageal reflux. This system works by suctioning the gastric wall and suturing it through a continuous and cross-linked fashion making a tube-like shape of the stomach. The excess weight loss observed at 1 year in a small study of 64 patients was about 58% (Fogel et al. 2008).

The Overstitch Endoscopic Suturing System (Apollo Endosurgery, Austin, TX, USA) permits full-thickness suturing of the gastric wall by using a flexible double-channel endoscope. A meta-analysis on 1859 patients showed a per cent weight loss of 16.43% (95%CI: 15.23–17.63) and 20.01% (95%CI: 16.92–23.11) at 12 and 24 months, respectively (Singh et al. 2020). While mortality was absent, the pooled incidence of serious adverse events was 2.26% (95%CI 1.25–4.03). The incidence of bleeding was 0.82% (95% CI 0.49–1.38), that of perforation 0.54% (95% CI 0.22–1.34, 2 studies), that of severe abdominal pain 0.68% (95% CI 0.38–1.20) and the pooled incidence of pulmonary embolism was 0.48% (95% CI 0.19–1.25).

Primary obesity surgery endoluminal (POSE) procedure uses the incisionless operating platform (USGI Medical, San Clemente, CA, USA). In a multicentre randomized controlled trial, POSE combined with lifestyle modification for 1 year resulted in a per cent weight loss of $4.95 \pm 7.04\%$ as compared to $1.38 \pm 5.58\%$ in the sham group ($n = 111$). Serious adverse event occurred in 4.7% of the patients and were related to gastro-intestinal symptoms including vomiting in 1.9% of the cases, nausea in 1.6% and pain in 0.4% (López-Nava et al. 2015).

The Endomina triangulation platform (Endo Tools Therapeutics SA – ETT, Gosselies, Belgium) permits to perform transmural sutures with serosa-to-serosa apposition. In a recent study on 71 patients the mean EWL at 6 months was significantly higher in the intervention (38.6%, $n = 45$) than in the control group (13.4%, $n = 21$; $P < 0.001$). At 1-year follow-up the EWL after Endomina was 45.1%, corresponding to a total body weight loss of 11.8%. Transient abdominal cramps occurred in 79.4% of the patients, transient nausea or mild vomiting in 66.2% (Huberty et al. 2020).

2.4 Incisionless Magnetic Anastomosis System

Using an incisionless magnetic anastomosis system under general anaesthesia, a partial jejunal diversion to the ileum was performed in 10 patients with obesity and

with or without type 2 diabetes in a first-in-human study. It was shown that the anastomosis remained widely patent at 1 year and that the total weight loss was 14.6% (40.2% EWL). The endoscopic operation improved also glycaemic control with an average glycated haemoglobin level reduction of 1.9%. No device-related serious adverse events occurred (Machytka et al. 2017).

2.5 Gastric Aspiration

The gastric aspiration system is performed under conscious sedation by placing endoscopically a gastrostomy tube (Atube) in the stomach, which is connected after a meal with the Aspire-Assist siphon assembly (Aspire Bariatrics, King of Prussia, PA, USA). This permits to suction about 30% of the ingested food with consequent reduction of energy intake.

In a small study on 25 subjects with obesity, EWL was $54.4 \pm 28.8\%$ ($P < 0.01$), effect that lasted 1 further year with an EWL of $61.5 \pm 28.5\%$ ($P < 0.01$) (Norén and Forssell 2016). Fifty-two per cent of the subjects reported pain and 9% severe pain. One of the 13 subjects had an intra-abdominal leakage at the gastrostomy site that required to be drained. Three subjects had adverse events during the first 30 days postoperative related to the stoma. One subject developed gallstones and pancreatitis.

2.6 Gastrointestinal Liner

The EndoBarrier (Fig. 2), a duodenal-jejunal bypass liner, consists of a 60 cm liner made of impermeable fluoropolymer. It is fixed in place on the duodenal bulb, distal to the pylorus, thanks to nitinol anchors with barbs. The gastric chime passes inside

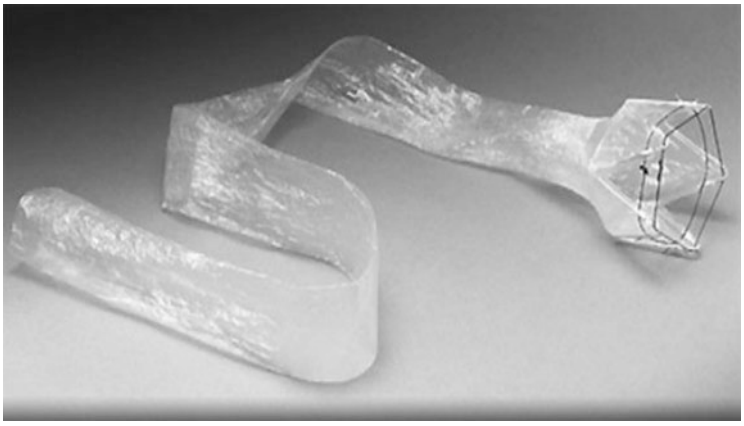

Fig. 2 Endobarrier. Proximal intestinal liner 60 cm long made out of impermeable fluoropolymer, its nitinol hooks with barbs which anchor to the duodenal mucosa just below the pylorus

the line while pancreatic juices and bile run along the outside of the sleeve mixing with nutrients distally to the sleeve end. This device was created with the aim of mimicking the RYGB in the bypass of the duodenum.

In fact, one of the hypotheses of the mechanism of action of RYGB in inducing diabetes remission, the so-called foregut hypothesis, is that the exclusion of the proximal small intestine from nutrient contact, decreases upper gut factors promoting insulin resistance and type 2 diabetes (Rubino et al. 2006; Castagneto Gissey et al. 2018b; Angelini et al. 2019).

A recent review of the literature and meta-analysis (Jirapinyo et al. 2018) found that the EndoBarrier determined a greater HbA1c reduction by 1.3% (95% CI 1.0–1.6, $P < 0.0001$) than pharmacotherapy alone after 8.4 ± 4.0 months from the implant. This effect persisted up to, at least, 6 months after the explant with a glycated haemoglobin change of 0.9% (95% CI 0.6–1.2). The weight loss change from baseline was 18.9% (95% CI 7.2–30.6] and excess weight loss was 36.9% (95% CI 29.2–44.6).

Insulin sensitivity greatly improved with a significant decrease of HOMA-IR by 4.6 (CI 2.9–6.3, $P < 0.0001$).

A recent prospective study with 3 year follow-up (Quezada et al. 2018) showed that 68% of the patients presented 72 severe adverse events, 55 of them device-related, requiring hospital admission. Eleven per cent of the patients required a prolonged hospitalization due to a liver abscess (4%), upper GI bleeding (5%), cholangitis (1.2%) and acute pancreatitis (1.2%).

2.7 Duodenal Mucosa Resurfacing

Hydrothermal duodenal mucosal resurfacing (DMR) (Fractyl Laboratories, Lexington, MA, USA) (Fig. 3) is a procedure targeting type 2 diabetes and non-alcoholic fatty liver disease, in which thermoablation of a portion of the duodenal mucosa is performed endoscopically. In the first in-human study, the length of the ablated duodenal segment was variable (Rajagopalan et al. 2016). Thereafter, the procedure was optimized with circumferential ablation of 3–15 cm of the post-papillary duodenal mucosa.

In the REVITA-2, a double-blind, superiority randomized controlled trial investigating safety and efficacy of DMR, the effect of this procedure at 24 weeks

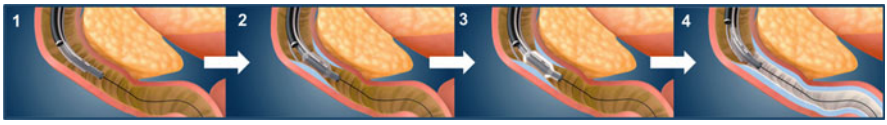

Fig. 3 Duodenal mucosal resurfacing (DMR) procedure. (1) The Revita DMR catheter is placed under deep sedation in the proximal duodenum distal to the papilla using a guidewire. (2) The balloon is then inflated and the intestinal mucosa is aspirated inside the balloon under vacuum condition. Saline solution is delivered to lift the mucosa. (3) Hot water is circulated into the balloon to ablate the previously expanded tissue and cycles repeated (4)

on HbA1c was not superior to sham in the overall population (−10.4 mmol/mol (1.0%) in the DMR group compared with −7.1 mmol/mol (0.7%) in the sham group, $P = 0.147$). However, a significant heterogeneity between the Brazil and European population was observed and the results were stratified by region. In the European population, DMR determined a significant reduction of HbA1c compared with the sham operation (6.6 ± 17.5 mmol/mol vs. 3.3 ± 10.9 mmol/mol, $P = 0.033$). Also non-alcoholic fatty liver disease was ameliorated with a reduction of the liver fat content investigated at 12 weeks after the procedure by MRI proton-density −5.4 ± 6.1% versus −2.2 ± 4.3% post-sham ($P = 0.035$).

The weight loss was 3 kg on average and the overall serious adverse event (SAE) rate was 2.5% with adverse events mostly mild and transient (Mingrone et al. 2021b).

According to the American Society for Gastrointestinal Endoscopy (ASGE) position statement on endoscopic bariatric therapies (ASGE Bariatric Endoscopy Task Force et al. 2015), endoscopic bariatric therapies (EBT) should be considered for patients with:

- Failed weight loss or weight maintenance with lifestyle intervention alone, unless medical conditions exist that require earlier addition of adjunctive therapy
- BMI criteria for primary EBT (this may vary with individual EBTs)
- Medical conditions that require weight loss for additional therapy but may exceed BMI criteria for primary EBT (bridge therapy)

Therefore, the eligibility criteria for endoscopic bariatric therapy are vague even for the BMI. More stringent criteria should be proposed in addition to preoperative workup and postoperative follow-up recommendations, otherwise these procedures will remain in limbo.

Endoscopic procedures for weight loss might be used as a bridge for bariatric surgery or as a standing alone therapy in less severe cases of obesity. However, scientific societies should develop guidelines with specific indications for endoscopic bariatric therapies.

3 New Anti-obesity Medications

New anti-obesity medications show remarkable effects on weight loss, somewhat comparable with laparoscopic and/or endoscopic surgery.

Twenty weeks of once-weekly subcutaneous administration of escalation doses of cagrilintide from 0.16 to 2.4 mg together with semaglutide 2.4 mg resulted in mean bodyweight changes ranging from −8.3% (SE 1.6; −8.0 kg [SE 1.5]) to −17.1% (1.5; −15.9 kg [1.4]) (Enebo et al. 2021).

Although tirzepatide, a dual glucose-dependent insulinotropic polypeptide and glucagon-like peptide-1 receptor agonist administered once a week subcutaneously, was developed to treat type 2 diabetes, the weight loss observed was greater with tirzepatide than with semaglutide (least-squares mean estimated treatment

difference, −1.9 kg, −3.6 kg and − 5.5 kg, respectively; $P < 0.001$ for all comparisons) (Frías et al. 2021). Therefore, it is possible that higher doses of tirzepatide can be effective in treating obesity.

Many other molecules are in the drug discovery pipeline for obesity, but this is the topic of a dedicated chapter of this book.

4 Conclusions

Maintaining weight loss in the long term is very challenging with lifestyle modifications. A meta-analysis including 29 long-term weight loss studies demonstrated that more than half of the lost weight was regained within 2 years, and that by 5 years after starting the dietary intervention more than 80% weight loss was regained (Anderson et al. 2001). In contrast, laparoscopic bariatric/metabolic surgery allows a conspicuous weight reduction durable over decades. Moreover, it is associated with a series of beneficial effects on obesity-related comorbidities, including a substantial improvement of glycaemic control in subjects affected by type 2 diabetes up to diabetes remission, improvement of blood pressure control, reduction of cardiovascular risk with prevention of cardiac infarction and stroke, and increased life expectancy. However, laparoscopic surgery is also associated with mortality, which indeed is even lower than mortality for elective cholecystectomy (Böckelman et al. 2017), and morbidity not only associated with surgery but also related to vitamin deficiency and, in malabsorptive interventions such as biliopancreatic diversion and SADI-S, protein deficiency.

Endoscopic bariatric procedures are less invasive, but the weight loss observed is less impressive and not durable over time, the same for the effects on diabetes control and insulin resistance improvement.

Understanding the role of the upper gut in the amelioration of insulin resistance and beta-cell glucose sensitivity as well as on satiety control can permit to find new pharmacological avenues to treat type 2 diabetes and obesity, which could be comparable to bariatric/metabolic surgery in the future.

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Drugs for Treating Obesity

Donna H. Ryan

Contents

Abstract

Older medications approved for chronic weight management (orlistat, naltrexone/bupropion, liraglutide 3 mg and, in the USA, phentermine/topiramate) have not been widely adopted by health care providers. Those medications produce only modest additional weight loss when used to augment lifestyle intervention. However, semaglutide 2.4 mg weekly has recently emerged and produces much more weight loss – on average 15% weight loss at 1 year. Semaglutide's

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enhanced efficacy and that its class (GLP-1 receptor analogs) is well-known may result in more clinicians adopting pharmacotherapy. Furthermore, the first dedicated cardiovascular outcome trial powered for superiority testing an anti-obesity medication (SELECT) is underway with semaglutide 2.4 mg. A positive outcome will further promote the concept that weight management should be a primary target for cardiometabolic disease control. In phase 3, tirzepatide and cagrilintide/semaglutide combination are showing promise for even greater weight loss efficacy. Another recently approved medication takes a personalized medicine approach; setmelanotide is approved as a therapy for those with some of the ultra-rare genetic diseases characterized by severe, early onset obesity. This chapter reviews the currently available and anticipated medications for chronic weight management as well as those approved for the genetic and syndromic obesities.

Keywords

Anti-obesity medication · Bimagrumab · Cagrilintide · Chronic weight management · Liraglutide · Naltrexone/bupropion · Obesity drugs · Obesity pharmacotherapy · Orlistat · Phentermine/topiramate · Semaglutide · Setmelanotide · Tirzepatide

1 Introduction and Rationale for Anti-obesity Medications

Obesity is increasingly being recognized as the root cause of the growing global burden of non-communicable diseases (NCDs), such as diabetes, cardiovascular diseases, and cancers. The NCDs stress societies' health systems, both economically and logistically. Thus, reducing excess abnormal fat has become a target for chronic disease prevention and remediation. In March 2021, the European Commission issued a brief in which it defined obesity as a "chronic relapsing disease, which in turn acts as a gateway to a range of other non-communicable diseases.". To effectively achieve and sustain weight loss in persons with obesity, it must be recognized as a disease, a chronic disease.

To address obesity as a disease, development of safe and effective pharmacologic agents is an imperative. When medications for weight management are used to augment lifestyle intervention targeting diet and physical activity, they offer potential as a rational pathway to cardiometabolic disease treatment and prevention, because they will provide more weight loss than can be achieved with lifestyle intervention alone.

Rationale for Anti-obesity Medications and for Long-Term Use Medications work through biology to promote greater adherence to consumption of fewer calories. In the case of orlistat, the mechanism is through blocking absorption of dietary fat and promoting adherence to low-fat meals and snacks (Guerciolini 1997). In the case of naltrexone/bupropion, liraglutide, phentermine/topiramate, and semaglutide, the mechanism is through appetite – reducing hunger, increasing

satiation, and making the patient less susceptible to highly hedonic foods (Pilitsi et al. 2019). As will be demonstrated in the discussions below, patients who take medications approved for chronic weight management will lose more weight than those taking placebo and as long as the medication is continued weight regain will be avoided. When the medications are stopped, weight is then regained. This is because obesity is like other chronic diseases. In the case of obesity, the body's defense of its highest fat mass drives weight regain through biologic effects on appetite regulation and energy expenditure regulation (Laughlin et al. 2021).

Indications for Anti-obesity Medications Regulatory authorities in the United States of America (USA) and European Union (EU) currently provide labeling that indicates anti-obesity medications are indicated for adults with BMI >30 kg/m^2 or >27 kg/m^2 and at least one comorbidity as an adjunct to reduced calorie diet and increased physical activity when the patients are unsuccessful in losing weight with lifestyle changes alone, or need to lose 10% or more body weight to achieve health benefits, or need to maintain weight loss (regardless of the methods used to achieve initial weight loss). As genetic defects and endocrine syndromes are discovered, indications are being granted for specific therapies. We now have treatment for defects in leptin, the leptin receptor, POMC, and PCSK1.

How Much Weight Loss Is Needed? For years, obesity medicine specialists have promoted the benefits of modest weight loss (5–10%), in part because that is all that can be achieved in most patients using older therapeutic approaches, excepting bariatric surgery. Modest weight loss (5–10%) is associated with improvement in glycemia, cardiovascular risk factors like blood pressure and lipids, and improvements in how patients feel and function (Ryan and Yockey 2017). However, greater amounts of weight loss (>10%) produce continued improvement in these outcomes. Further, 10% or more weight loss is needed for improvement in symptoms of obstructive sleep apnea (OSA) and for improvements in NASH Activity Scores in patients with Non-Alcoholic Steatotic Hepatitis (NASH) (Ryan and Yockey 2017). For diabetes remission, 15 kg weight loss is needed; (Lean et al. 2018, 2019) and for reduction in cardiovascular events, 15% or more weight loss is probably needed (Ryan and Yockey 2017). Different amounts of weight loss produce different effects on different tissues (Magkos et al. 2016). Visceral and ectopic fat stores are mobilized preferentially (Magkos et al. 2016), and this may account for the metabolic improvements with more modest weight loss, while greater weight loss is required for other conditions.

Variation in Weight Loss Response An important observation in weight management is that no matter what treatment we are initiating, there is enormous individual variation in weight loss response (MacLean et al. 2018). This is true for all our treatments, including surgery and medications. The implications of this for prescribers is that for all medications, early response is predictive of long-term outcomes.

2 Anti-obesity Medications Recently Brought to Market: Semaglutide 2.4 mg and Setmelanotide

2.1 Semaglutide 2.4 mg

Semaglutide is an analog of native GLP-1 (glucagon-like peptide-1) and has 94% homology with the native peptide sequence. In semaglutide, arginine replaces lysine at position 28, aminoisobutyric acid replaces glycine at position 2 (to resist degradation) and a C-18 fatty acid and lengthy spacer is attached to Lysine (to promote albumen binding) (Pearson et al. 2019). While native GLP-1 has a half-life of 1–2 min, the half-life of semaglutide is 165 h, allowing it to be dosed subcutaneously once weekly (Pearson et al. 2019). Thus, semaglutide 2.4 mg is given weekly by subcutaneous injection. The dose escalation schedule is to increase every 4 weeks from 0.25 mg to 0.5 mg, to 1.0 mg, to 1.7 mg, to 2.4 mg (Wegovy™ Product Label, FDA).

GLP-1 receptor analogs have pleiotropic effects (Ryan and Acosta 2015). There are multiple agents in this class approved for type 2 diabetes, but liraglutide (discussed below) is the only GLP-1 analog approved for weight management. Semaglutide is approved for management of diabetes at doses of 0.5 and 1.0 mg weekly and oral semaglutide in doses up to 14 mg is also approved for diabetes. Semaglutide 0.5 and 1.0 mg have been shown to reduce cardiovascular events in persons with type 2 diabetes (Marso et al. 2016).

Semaglutide is approved in the USA and is under review by the European Medicines Agency (EMA). Five phase 3 studies, all called STEP (Semaglutide Treatment Effect in People with obesity) are now completed; (Kushner et al. 2020) four have been published (Wilding et al. 2021; Davies et al. 2021; Wadden et al. 2021; Rubino et al. 2021). In these studies, a "treatment policy estimand" was used for the primary analysis. This is like an intention-to-treat analysis where all assigned participants are considered, and missing data are accounted for with statistical measures of multiple imputation. Another "trial product estimand" was calculated which considered observations on treatment. This review will report the more conservative "treatment policy estimand" for the discussion of results across trials, except where noted.

The characteristics of the four STEP trials are shown in Table 1. In STEP 1, more than 70% patients had a comorbidity and while none had diabetes, almost 44% had prediabetes. Both placebo and semaglutide 2.4 mg groups received a lifestyle intervention with a 500 kcal/day deficit diet and recommendations to increase physical activity to 150 min per week. The trajectory of mean weight loss in this study was such that the mean weight loss did not reach a plateau until 60 weeks see Fig. 1a. In STEP 1, when the semaglutide-treated group is compared to the placebo-treated group, there were greater improvements in cardiometabolic risk factors and a greater increase in participant-reported physical functioning.

STEP 2 (Davies et al. 2021) enrolled 1,210 persons with type 2 diabetes and randomized them 1:1:1 to semaglutide 2.4 mg weekly, semaglutide 1.0 mg weekly, or placebo. The weight loss trajectory for semaglutide 2.4 and 1.0 mg was like that in

Table 1 Characteristics of four recently published phase 3 studies of semaglutide 2.4 mg weekly for obesity

	STEP 1 (Wilding et al. 2021)	STEP 2 (Davies et al. 2021)	STEP 3 (Wadden et al. 2021)	STEP 4 (Rubino et al. 2021)
Population	1,961 adults with BMI ≥30 or BMI ≥27 kg/m^2 with ≥1 weight-related comorbidity, without diabetes enrolled at 129 sites in 16 countries	1,210 adults with BMI ≥27 kg/m^2 with type 2 diabetes enrolled at 149 clinics in 12 countries	611 adults with BMI ≥30 or BMI ≥27 kg/m^2 with ≥1 weight-related comorbidity, without diabetes enrolled at 41 sites in the United States	902 adults with BMI >30 or BMI >27 kg/m^2 with >1 weight-related comorbidity entered 20-week run-in; 806 reached 2.4 mg dose semaglutide and entered randomization; 73 sites in 10 countries
Randomization scheme	Randomized 2:1 to 2.4 mg semaglutide vs. placebo	Randomized 1:1:1 to 2.4 mg semaglutide vs. 1.0 mg semaglutide[a] vs. placebo	Randomized 2:1 to 2.4 mg semaglutide vs. placebo	At week 20, those who achieved 2.4 mg dose semaglutide randomized 2:1 to continued 2.4 mg semaglutide vs. placebo
Drug treatment scheme	Prefilled pens; initial semaglutide dose 0.25 mg subcutaneous Once weekly for first 4 weeks; semaglutide dose increased every 4 weeks to reach 2.4 mg; treatment duration 68 weeks	Prefilled pens for 2 injections once a week; initial semaglutide dose 0.25 mg subcutaneous Once weekly for first 4 weeks; semaglutide dose increased every 4 weeks to reach 2.4 mg; treatment duration 68 weeks	Prefilled pens; initial semaglutide dose 0.25 mg subcutaneous Once weekly for first 4 weeks; semaglutide dose increased every 4 weeks to reach 2.4 mg; treatment duration 68 weeks	First 20 weeks, open-label treatment with once-weekly subcutaneous Semaglutide, 0.25 mg, increased every 4 weeks to the maintenance dose of 2.4 mg by week 16, and Continued to week 20. Then, randomized to pre-filled pens with placebo or semaglutide 2.4 mg weekly for double-blind therapy
Background treatment	Both groups received lifestyle intervention: 500 kcal/day deficit diet and increased physical activity to 150 min/ week	All groups received lifestyle intervention: 500 kcal/day deficit diet and increased physical activity to 150 min/ week	Both groups received low-calorie diet for 8 weeks followed by intensive behavioral therapy (i.e., 30 counseling visits)	Both groups received lifestyle intervention: 500 kcal/day deficit diet and increased physical activity to 150 min/ week

(continued)

Table 1 (continued)

	STEP 1 (Wilding et al. 2021)	STEP 2 (Davies et al. 2021)	STEP 3 (Wadden et al. 2021)	STEP 4 (Rubino et al. 2021)
Primary end point(s)	Percentage change in body weight and weight reduction of at least 5% at week 68	Percentage change in body weight and weight reduction of at least 5% at week 68	Percentage change in body weight and weight reduction of at least 5% at week 68	Percentage change in body weight from week 20 to week 68
Trial completion rate	93.4%	96%	92.8%	98.0% of randomized
Treatment adherence rate	81.1%	87%	82.7%	92.3% of randomized
Baseline characteristics	74.1% female 75.1% white Mean age 46 years Mean weight 105.3 kg Mean BMI 37.9 43.7% had prediabetes 70.5% had one or more coexisting conditions	50.9% female 62.1% white Mean age 55 years Mean weight 99.8 kg Mean BMI 35.7 Mean HbA_{1c} 8.1% Biguanide drug use in 91.8%	81.0% female 76.3% white Mean age 46 years Mean weight 105.8 kg Mean BMI 38.0 74.1% had one or more comorbidity at screening	79% female 83.7% white Mean age 46 years Mean weight 107.2 kg Mean BMI 38.4 64.8% had 1–3 comorbidities
Mean change in body weight at week 68				
Semaglutide 2.4 m	−14.9%	−9.6%[a]	−16.0%	−7.9% from week 20 −17.4% from week 0
Placebo	−2.4%	−3.4%	−5.7%	+6.9% from week 20 −5.9% from week 0
Proportion achieving >5% weight loss at week 68				
Semaglutide 2.4 mg	86.4%	68.8%	86.6%	88.7% from week 0
Placebo	31.5%	28.5%	47.6%	46.6% from week 0
Proportion reporting serious adverse events				
Semaglutide 2.4 mg	9.8%	9.9%	9.1%	7.7%
Placebo	6.4%	9.2%	2.9%	5.6%

Proportion discontinuing because of adverse events				
Semaglutide	7.0%	6.2%	5.9%	2.4%
Placebo	3.1%	3.5%	2.9%	2.2%

[a]Body weight change at week 68 was −6.99% for semaglutide 1.0 mg weekly

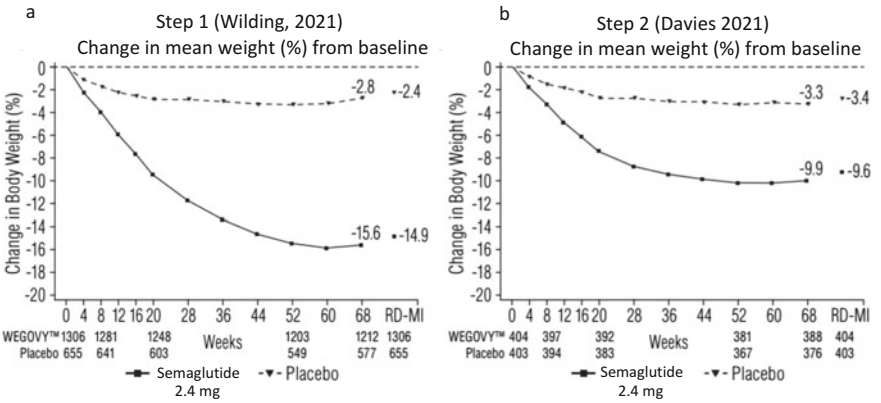


Fig. 1 (**a**, **b**) Step 1 and Step 2 Trials. In these studies, all individuals are given the same lifestyle intervention and randomized to placebo or semaglutide 2.4 mg subcutaneous weekly. Trajectories represent observed data (trial product estimand). Also shown are the intention to treat endpoint (treatment policy estimand. The visual data are publicly available in the US product label at https://www.accessdata.fda.gov/drugsatfda_docs/label/2021/215256s000lbl.pdf

STEP 1, except the mean weight losses for semaglutide 2.4 mg in STEP 2 were lesser than those in STEP 1 at the same dose, albeit greater than semaglutide 1.0 mg or placebo see Fig. 1b. One of the coprimary endpoints was percent weight loss at 68 weeks for semaglutide 2.4 mg vs. placebo. Mean change in body weight was −9.6% at week 68 for semaglutide 2.4 mg and −3.4% for placebo, with an estimated treatment difference of 6.21% [CI 7.28 to 5.15]; $P < 0.0001$. For the semaglutide 1.0 mg treatment group mean weight loss at week 68 was −7.0% at week 68.

The mean weight loss in STEP 2 is less than that in STEP 1. The background lifestyle intervention follows the same protocol in both studies, but the populations differ; STEP 2 consists of persons with type 2 diabetes and there were none in STEP 1 and the mean weight loss observed in persons with diabetes is always less than those without diabetes. There was biguanide use in 91.8% of enrolled persons in STEP 2 and the protocol called for a 50% dose reduction of biguanide medication at study start. In Look AHEAD, a lifestyle intervention that produced 9.6% weight loss at 52 weeks, there was a personalized protocol for stopping or reducing diabetes medications, whereby persons with acceptable diabetes control at baseline had medications stopped at the start of the dietary intervention (Look AHEAD Research Group 2006).

In STEP 3, (Wadden et al. 2021) enrolled participants were randomized to semaglutide 2.4 mg or placebo. Both groups received an intensive behavioral intervention which consisted of an initial 8-week low-calorie diet (1,000–1,200 kcal/day) provided as meal replacements. Then, this highly structured diet was transitioned to a 1,200–1,800 kcal/day of conventional food for the remainder of the 68 weeks. Physical activity began with 100 min of physical activity per week and increased by 25 min every 4 weeks to ultimately 200 min per week. The

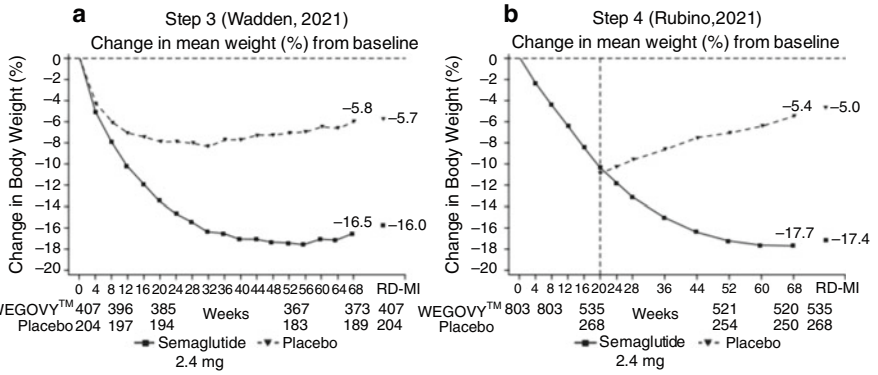


Fig. 2 (**a**) STEP 3. Participants were randomized to semaglutide 2.4 mg or placebo. Both groups received an intensive lifestyle intervention (see text). The weight loss in the placebo group illustrates the effect of the more intensive intervention. (**b**) STEP 4. During the first 16 weeks, all patients receive semaglutide dose escalation to 2.4 mg. Those achieving this dose (92%) were randomized to placebo or semaglutide 2.4 mg. For both studies, trajectories represent observed data (trial product estimand). Also shown are the intention to treat endpoint (treatment policy estimand. The visual data are publicly available in the US product label at https://www.accessdata.fda.gov/drugsatfda_docs/label/2021/215256s000lbl.pdf

mean weight loss in this study with placebo reflects the greater intensity of the lifestyle intervention; placebo-treated participants lost on average −5.7% at week 68 see Fig. 2a. Although not head-to-head comparisons, this is greater than the mean weight loss of −2.4% in a similar population in STEP 1 (See Fig. 1a.) who received a similar intervention. However, the mean weight loss in the semaglutide 2.4 mg treatment group was −16.0% (see Fig. 6); this is slightly greater than STEP 1 (−14.9%). The estimated treatment differences in mean weight loss at 68 weeks between placebo and semaglutide 2.4 mg were −12.4% in STEP 1 and −10.3% in STEP 3.

STEP 4 (Rubino et al. 2021) was designed to show the long-term impact over 48 weeks of continuing semaglutide after reaching the 2.4 mg dose at 20 weeks see Fig. 2b. All participants received semaglutide open label during a dose escalation period over 16 weeks and then the dose was continued for 4 weeks. Of the 902 individuals who enrolled, 806 (92%) reached the 2.4 mg dose and were randomized to placebo or continued semaglutide 2.4 mg. Those who continued semaglutide after randomization continued to lose weight reaching a plateau at week 60 to week 68 and ultimately achieving −17.4% weight loss from entry. In comparison, those on placebo gradually regained weight see Fig. 2b. The weight loss with semaglutide 2.4 mg was associated with improvements in cardiometabolic risk factors in this study.

The safety and tolerability across STEP 1, 2, 3, and 4 (Wilding et al. 2021; Davies et al. 2021; Wadden et al. 2021; Rubino et al. 2021) demonstrated the predicted findings with this drug and class. In all studies, gastrointestinal disorders (typically nausea, diarrhea, vomiting, and constipation) were the most frequently reported

events and occurred in more participants receiving semaglutide than those receiving placebo. Most gastrointestinal events were mild to moderate in severity, were transient, and resolved without permanent discontinuation of the regimen. Gallbladder-related disorders (mostly cholelithiasis) were reported more often in STEP 1 and STEP 3. In STEP 1, gallbladder disorders occurred in 2.6% and 1.2% of participants in the semaglutide and placebo groups, respectively (Wilding et al. 2021). In STEP 3, gallbladder-related disorders (mainly cholelithiasis) were reported in 4.9% of semaglutide-treated participants and 1.5% of those on placebo (Wadden et al. 2021). Acute pancreatitis also occurred in small numbers in semaglutide-treated patients (3 in STEP 1, 1 in STEP 2, 0 in STEP 3, and 1 in STEP 4) (Wilding et al. 2021; Davies et al. 2021; Wadden et al. 2021; Rubino et al. 2021). Overall, there were no unexpected safety findings in the reports of the four trials.

The chief safety issues with drugs of this class are the rare occurrence of pancreatitis and a prohibition of use in patients with a personal or family history of Multiple Endocrine Neoplasia Type 2 or medullary thyroid carcinoma. Semaglutide 1.0 mg weekly has been shown to reduce cardiovascular events in persons with diabetes and other GLP-1 receptor agonists have also demonstrated cardioprotection (Marso et al. 2016). Prescribers' confidence in semaglutide for obesity will likely increase if the ongoing SELECT study (Ryan et al. 2020) demonstrates that semaglutide 2.4 mg weekly is associated with reduction of cardiovascular events in persons with overweight and obesity and who have pre-existing cardiovascular disease.

The aim of weight management should be normalization of body composition, not just reducing weight. In STEP 1, Dual Emission X-ray Absorptiometry data were reported on a subset of participants ($N = 140$) (Wilding et al. 2021). In that substudy, there was mean loss of −8.36 kg of total body fat mass and −5.26 kg of total body lean mass in the semaglutide-treated participants. In the placebo group the mean loss was −1.37 kg fat mass and −1.83 kg lean mass. The usual proportion lean loss in total weight loss is 25% (Heymsfield et al. 2014). It is important to reduce excess abnormal fat mass, without adversely affecting muscle and bone. Look AHEAD, a study comparing intensive lifestyle intervention (ILI) to diabetes support and education (DSE) in persons with type 2 diabetes is informative in showing that not all persons experience only health benefits from weight loss; there are some negative outcomes (Wing and Look AHEAD Research Group 2021). As expected with weight loss, ILI led to greater reductions in fat mass than DSE, but also greater loss of lean body mass during active weight loss and when ILI participants regained weight, they regained mainly fat mass (Pownall et al. 2015). In addition, there were greater decreases in bone density for both total hip (−1.4% vs. −0.4%, $P < 0.001$) and femoral neck (−1.5% vs. −0.8%; $P < 0.009$) in ILI vs. DSE at 1 year (Schwartz et al. 2012). The relationship to hip fracture in Look AHEAD is uncertain. The risk for hip fracture was elevated in ILI compared to DSE (HR = 1.78 [95% CI 0.98, 3.25] $P = 0.06$), but this finding was not statistically significant (Johnson et al. 2017). It cannot be determined with accuracy from DEXA what the loss of muscle mass or bone mass might be. But this issue deserves further study with more advanced techniques to measure body composition changes. Meanwhile, we will

need to reinforce the importance of weight bearing exercise and strength training in patients who are losing weight with semaglutide and use caution in patients with sarcopenic obesity.

2.2 Setmelanotide

Setmelanotide Setmelanotide is a cyclized octapeptide that binds and activates multiple melanocortin receptors – MC4R, MC3R, and MC1R selectively over MC5R and MC2R (Sharma et al. 2019). Setmelanotide is one of Multiple MC4R agonists that have been studied as potential anti-obesity medications (Sharma et al. 2019). Some of these activate the sympathetic nervous system with blood pressure elevation and increased heart rate making them unacceptable in clinical care, while setmelanotide has not been shown to have this characteristic (Sharma et al. 2019). In a diet-induced obese nonhuman primate model, setmelanotide produced persistent weight loss (−13.5%) over 8 weeks (Kievit et al. 2013). Importantly, it did not increase heart rate or blood pressure. In a phase 1b study in humans, individuals with obesity and heterozygous for complete or partial loss of function mutations in MC4R were treated with setmelanotide by infusion or placebo over 28 days (Collet et al. 2017). Interestingly, both groups lost weight similarly, in comparison with placebo. There were no increases in heart rate or blood pressure in this study, but the most frequent side effect was skin darkening, or "tanning" associated with setmelanotide (Collet et al. 2017). This early study demonstrated that there would probably be limited advantage for setmelanotide in heterozygous individuals, although depending on functional variants, different responses might be obtained. The clinical development of the drug then focused on identifying homozygous individuals with genetic defects that might respond to setmelanotide.

Setmelanotide was developed with a personalized medicine approach, targeting the drug for individuals with defects in the melanocortin pathway. Setmelanotide showed excellent outcomes in two patients with POMC deficiency, reversing hyperphagia and producing dramatic weight loss in both patients (Kühnen et al. 2016). When given to three patients with LEPR deficiency, setmelanotide produced clinically significant reduction in both body weight and hyperphagia (Clément et al. 2018). The drug has also been studied in 7 patients with Bardet-Biedl syndrome, showing hunger reduction and mean weight loss at 1 year of −16.3% (90% CI, −19.9% to−12.8%; $n = 7$) (Haws et al. 2020). Bardet-Biedl continues to be studied as potential indication for setmelanotide.

The regulatory approval of setmelanotide rests on a study (Clément et al. 2020) in 21 participants (Imcivree™ Product Label, FDA 2021; Imcivree™ product label, EMA 2021), where the genetic defects were biallelic variations in either the prohormone, pro-opioid melanocortin (POMC) ($n = 9$), PCSK1 (proprotein convertase subtilisin and kexin type 1) ($n = 1$), an important enzyme in activating the melanocortin 4 receptor pathway, or the leptin receptor (LEPR) ($n = 11$), which is essential for POMC function. The study was designed with a variable period of dose-finding where the drug was administered daily, and dose adjusted to manage

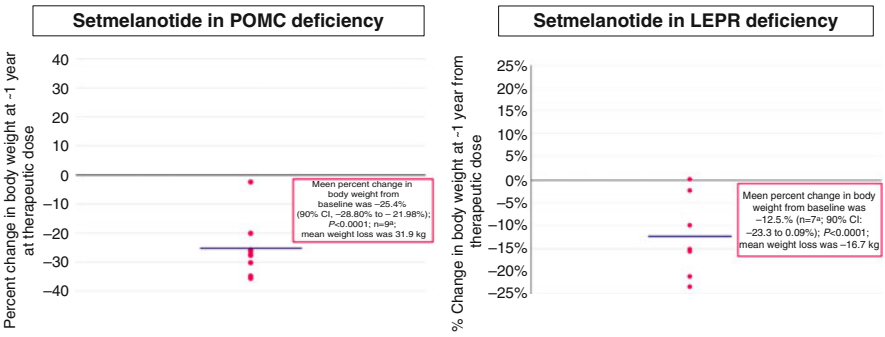


Fig. 3 In this study, the drug was administered daily, starting at 1 mg to patients with homozygous genetic deficiency in POMC, PCSK1, and Leptin Receptor. There was maximum 32 weeks of open-label therapy for responsive patients. Note: Y axis scales are not identical. Visual data publicly available at https://rhythmpharmaceuticals.gcs-web.com/static-files/bc6550a7-a5df-4d9a-9a81-fcf41ba95066

hyperphagia. Then a 10-week open-label period occurred, and participants were required to lose 5 kg or 5% if the body weight was less than 100 kg to continue the study. Successful patients entered an 8-week placebo-controlled phase inclusive of a 4-week placebo period and then continued for 32 weeks of open-label therapy.

The study (Clément et al. 2020) showed that for the 10 patients with POMC or PCSK1 deficiency, 8 of 10 met the primary outcome of 10% or more weight loss at 1 year; among all enrollees, mean weight loss was −25.6%. These results are shown in Fig. 3, *below*. For the 11 patients with LEPR deficiency, the response was more variable. Of those 11, four failed to achieve the required 5% weight loss by week 12 and only five (45%) achieved the primary outcome of 10% or more weight loss at 1 year (Clément et al. 2020). Still, all five achieved 15% or more weight loss and two achieved 20% or more weight loss (Clément et al. 2020). These results are also shown in Fig. 3. For both LEPR and POMC deficiency patients, tolerability and safety seemed acceptable. The most common adverse events were injection site reactions, skin darkening and nausea, vomiting, and diarrhea (Clément et al. 2020). Other side effects included spontaneous penile erections and spontaneous female arousal, depression and suicidal thoughts and darkening of moles. Compared to those with LEPR deficiency, the results with setmelanotide were best for patients with POMC deficiency. We cannot be sure of the response in the one patient with PCSK1 deficiency since that patient had to drop out of study because the patient developed depression after hyperphagia recurred during a required blinded placebo phase (Clément et al. 2020). While the results were not as encouraging for all patients with LEPR deficiency as those with POMC deficiency in terms of amount of weight loss, this should be interpreted in the face of no alternative treatments for this severe disease.

Setmelanotide was approved by the US FDA (Imcivree™ Product Label, FDA 2021) with an indication for "chronic weight management (weight loss and weight maintenance for at least one year) in patients six years and older with obesity due to

three rare genetic conditions: pro-opiomelanocortin (POMC) deficiency, proprotein subtilisin/kexin type 1 (PCSK1) deficiency, and leptin receptor (LEPR) deficiency confirmed by genetic testing demonstrating variants in POMC, PCSK1, or LEPR genes considered pathogenic (causing disease), likely pathogenic, or of uncertain significance." The drug is priced at $330 per mg, making annual costs very high for this drug which requires daily subcutaneous injection and where doses begin at 1 mg (Imcivree™ Price 2021).

What does setmelanotide mean for the practice of obesity medicine? Regulatory approval has come only for patients with proven genetic defects in the leptin-melanocortin pathway. Having a drug that is effective would then drive clinicians to increase genetic testing for patients with a history of severe early onset obesity. Thus, the impact of setmelanotide in the obesity clinic is likely to mean a renewed appreciation for the biologic underpinnings of obesity and an increase in genetic screening to identify a subset of patients. Still, the three genetic conditions for which setmelanotide has been approved are ultrarare. They are associated with severe childhood obesity and hyperphagia and may be associated with various other endocrinopathies, e.g., adrenocorticotropic hormone deficiency, hypothyroidism, hypogonadism, hypopigmentation, hypoglycemia, and others. The number of individuals in the USA proposed to have genetic mutations in the melanocortin pathway if we tested widely is estimated to be 12,800, a miniscule fraction of the population with obesity (Ayers et al. 2018). While they may occur only rarely, these conditions present enormous challenges for health care providers, parents, and patients. Thus, the primary users of setmelanotide are likely to be clinics where children with severe obesity are referred for evaluation. Practitioners must await guidance on adults – when and whom to test. Certainly, a history of early onset severe obesity would be the clinical presentation might stimulate genetic testing.

There will be efforts to identify other patients with other genetic obesity syndromes that might respond to setmelanotide. Setmelanotide is being tested in Bardet-Biedl syndrome and Alström syndrome in a Phase 3 trial (NCT03746522), as well as SRC1, SH2B1, and MC4R deficiency, and Smith-Magenis syndrome in a basket Phase 2 trial (NCT03013543). Still, this is unlikely to expand the user base for setmelanotide significantly. Given the global prevalence of obesity, the obvious question is, "Could setmelanotide have a broader indication for weight management?"

The data in nonhuman primates (Kievit et al. 2013) and in humans with obesity used as controls (Collet et al. 2017) demonstrate that there is some weight loss efficacy with setmelanotide in those without genetic melanocortin pathway defects. But the chief side effect, tanning, must be considered. That side effect might make the drug undesirable from a patient perspective. Will patients accept tanning if weight loss is robust? This question and other safety considerations could only be answered through the expensive and time-consuming drug development process requiring large patient numbers to establish safety and efficacy. That is not likely to happen and for now, setmelanotide is likely to remain solely in the realm of treatment for those with proven genetic defects in the melanocortin pathway. The

search for other indications will continue, however, with attempts to identify genotypes that would be highly responsive to this drug.

3 Older Anti-obesity Medications

3.1 Orlistat

Orlistat reversibly blocks the action of pancreatic and gastric lipases (Guerciolini 1997). Inactivation of these lipases prevents the hydrolysis of triglycerides, and thus free fatty acids are not absorbed. Orlistat is usually prescribed at 120 mg taken before meals three times daily and at this dose, 30% of dietary fat is not absorbed. Thus, orlistat has the effect of enforcing a low-fat diet, since foods or snacks high in fat will result in steatorrhea.

In a meta-analysis of 23 trials of patients with and without diabetes, orlistat plus intensive behaviorally based intervention produced weight loss of "5 to 10 kg (11 to 22 pounds), average, 8% of baseline weight, compared with 3 to 6 kg in the placebo groups" (Leblanc et al. 2011). The best study to demonstrate orlistat's efficacy is the 4-year double-blind, randomized, placebo-controlled trial (XENDOS Study) of lifestyle intervention with or without orlistat 120 mg three times daily in 3,304 patients with overweight or obesity (Torgerson et al. 2004) see Fig. 4. In that study, mean weight loss at 1 year was 11% with orlistat, but the orlistat-treated patients remained 6.9% below baseline at 3 years, compared with 4.1% for those receiving placebo. For those with impaired glucose tolerance (21% of the population), there was a reduction of 37% in the progression to type 2 diabetes with lifestyle

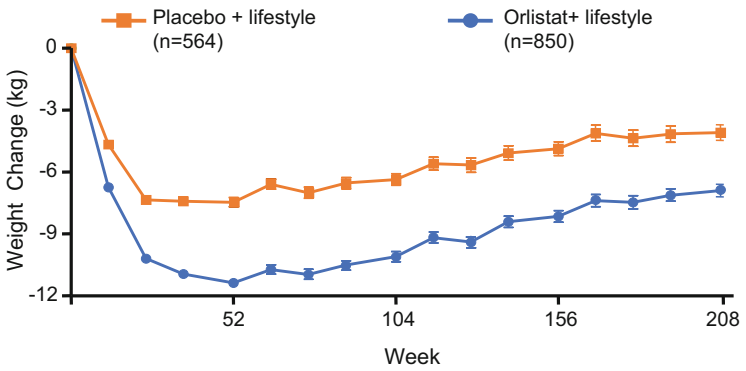


Fig. 4 Xendos Study Change in body weight (kg) are depicted as mean and SEM over 4 years (208 weeks) for patients receiving lifestyle intervention and randomly assigned to placebo or orlistat. Statistical analysis is by last observation carried forward (LOCF). Weight loss and maintenance is superior with orlistat when added to lifestyle intervention (image redrawn from Torgerson et al. 2004). $P < 0.001$. Baseline placebo + lifestyle = n 1637. Baseline orlistat + lifestyle = n 1640

intervention plus orlistat compared to lifestyle intervention plus placebo (Torgerson et al. 2004).

Orlistat is approved for weight management for adolescents in some countries. In 539 adolescents who received 120 mg three times per day of orlistat, on average, BMI decreased by 0.55 kg/m^2 in the drug-treated group compared to an increase of +0.31 kg/m^2 in the placebo-treated group (Chanoine et al. 2005).

Adherence to orlistat use falls off rapidly after initial prescription. In a Canadian study, the use of orlistat among 16,968 people initially started on this drug had fallen to 6% by 1 year and to only 2% by 2 years (Padwal et al. 2007). This may relate to the drug's tolerability profile, discussed below.

Orlistat is not absorbed from the GI tract to any significant degree, and its side effects relate to blockade of triglyceride digestion in the intestine (Guerciolini 1997). If orlistat is taken with a high fat meal or snack, then the effects of unabsorbed fat – steatorrhea –are likely to occur. Counseling patients about gastrointestinal side effects is important, so that patients can adhere to lower fat foods. It may also be helpful to take blond psyllium along with orlistat to minimize gastrointestinal side effects (Cavaliere et al. 2001). Because orlistat can cause small but significant decreases in fat-soluble vitamins some patients may need vitamin supplementation given at bedtime, particularly if it is continued long-term (McDuffie et al. 2002). Orlistat can reduce the absorption of some medications, notably cyclosporine, amiodarone, levothyroxine, anti-retroviral medications, and the lipophilic antiepileptics (Filippatos et al. 2008). It can also interfere with warfarin because of its action on Vitamin K (Filippatos et al. 2008). Orlistat has also been associated with calcium oxalate renal stones (Humayun et al. 2016).

3.2 Naltrexone SR/Bupropion SR

Bupropion has been used as a single agent for depression and for smoking cessation and is known to produce weight loss at 300 or 400 mg daily (Anderson et al. 2002). Naltrexone is an opioid receptor antagonist that is used for addiction to opioids or alcohol. It has minimal effect on weight loss on its own. Bupropion stimulates the POMC neuron which releases α-MSH and β-endorphin in the hypothalamus which stimulates feeding (Greenway et al. 2009). This effect on β-endorphin is blocked by naltrexone thus allowing the inhibitory effects of α-melanocyte stimulating hormone (α-MSH) to reduce food intake by acting on the melanocortin-4 receptor system (Greenway et al. 2009).

Efficacy of Naltrexone/Bupropion Three phase 3 studies with this combination provided the basis for its approval. In the COR I study (Contrave Obesity Research I) there were three treatment arms: placebo, NB 32/360 (32 mg of naltrexone and 360 mg of sustained release bupropion), and NB 16/360 (16 mg of naltrexone and 360 mg of sustained release bupropion). Using the primary analysis population in COR-I, the Least Squares (LS) mean percentage weight loss (SE) at 56 weeks was −1.3% (0.3) for the Placebo, −5.0% (0.3) for Naltrexone/Bupropion-16/360

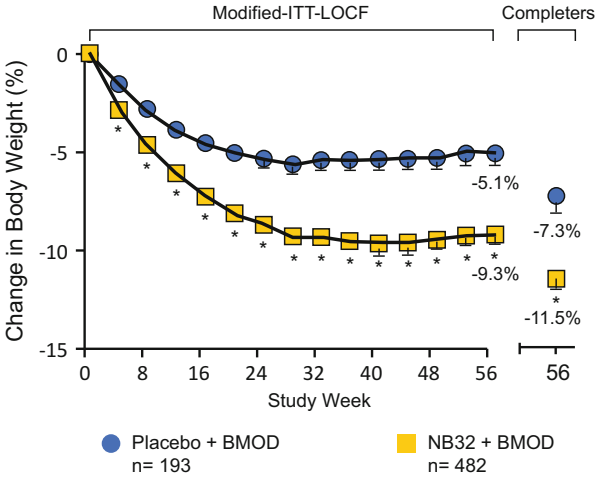


Fig. 5 COR/BMOD Study: Percent change in body weight over 56 weeks with intensive behavioral modification lifestyle intervention (BMOD) and randomized assignment to placebo or naltrexone 32 mg/bupropion 360 mg (NB32) (image redrawn from Wadden et al. 2011). The mean body weight for each study group is shown for the Modified-ITT-LOCF population across 56 weeks. The mean weight loss of completers is shown at week 56. $^{*}P < 0.001$, for NB32 + BMOD vs. placebo + BMOD. COR/BMOD = Contrave Obesity Research/Behavioral Modification; ITT = intent to treat; LOCF = last observation carried forward

(NB 16/360) ($P < 0.0001$ vs. placebo) and −6.1% (0.3) for NB 32/360 ($P < 0.0001$ vs. placebo) (Greenway et al. 2010). The NB16/360 and NB32/360 treatment arms had improvements in waist circumference, fasting glucose, fasting insulin, homeostasis assessment model of insulin resistance (HOMA-IR), HDL cholesterol, CRP, and Impact of Weight on Quality of Life – Lite (IWQOL-Lite) (Kolotkin et al. 2001) scores, when compared to placebo (Greenway et al. 2010).

As shown above in Fig. 5, below, COR-BMOD (Contrave Obesity Research-Behavior Modification) randomly assigned participants in a 1:3 ratio to either placebo (P) given with a behavior modification program (BMOD), or naltrexone sustained release 32 mg plus bupropion sustained release 360 mg (NB32/360) plus BMOD. The behavior modification program consisted of 28 group sessions, each of 90 min duration. The weight loss in COR BMOD was excellent for both the placebo and active treatment groups. At 56 weeks, mean weight loss in the P + BMOD group was 5.1 + 0.6% and for the NB 32/360 + BMOD group it was 9.3 + 0.4% ($P < 0.001$ vs. placebo +BMOD) (Wadden et al. 2011). There were significantly greater improvements in waist circumference, insulin, HOMA IR, HDL cholesterol, and triglycerides as for quality-of-life measurement, the scores on the IWQOL-Lite questionnaire improved significantly more in the group on active drug treatment than placebo (Wadden et al. 2011).

In COR II (Contrave Obesity Research II), participants were randomized 2:1 to combined naltrexone sustained release (SR) (32 mg/day) plus bupropion SR

(360 mg/day) (NB32) or placebo for up to 56 weeks. Significantly greater weight loss was observed with NB32 vs. placebo at week 56 (−6.4% vs. −1.2%) ($P < 0.001$) (Apovian et al. 2013). The weight loss was accompanied by improvements in cardiometabolic risk markers, weight-related quality of life, and a measure of control of eating (Apovian et al. 2013).

Finally, in patients with type 2 diabetes, use of the combination resulted in significantly greater weight reduction compared to placebo (5.0% vs. 1.8%; $P < 0.001$) and significantly greater reduction in HbA1c (−0.6 vs. −0.1%; $P < 0.001$) (Hollander et al. 2013). There was also improvement in triglycerides and HDL -cholesterol compared with placebo (Hollander et al. 2013).

Safety and Tolerability Profile of Naltrexone/Bupropion The chief tolerability issue with this medication is nausea, associated with the naltrexone component, which occurs on initiating the drug or escalating its dose (Contrave Product Label 2021; Mysimba Product Label 2021). While relatively common (about 30% of participants in the phase III studies) it accounted for <7% of dropouts (Contrave Product Label 2021). A dose escalation period of 4 weeks is used to minimize this side effect (Contrave Product Label 2021; Mysimba Product Label 2021). The drug should not be prescribed with concomitant use of SSRIs or MAOIs because of risk of serotonin syndrome with bupropion (Contrave Product Label 2021; Mysimba Product Label 2021).

The decline in blood pressure is not as great as one would expect from the weight loss in the Phase III trials of naltrexone/bupropion (Greenway 2010; Wadden et al. 2011). Bupropion is associated with an increase in pulse and both bupropion and naltrexone increase blood pressure. A required pre-marketing study of the combination drug with assessment of cardiovascular outcomes was subjected to an interim analysis (Nissen et al. 2016). This resulted in early study termination at the 50% interim analysis. Termination was due to inclusion of the 25% interim analyses on the patent publication, resulting in the potential for unblinding.

3.3 Liraglutide 3.0 mg

Liraglutide is a GLP-1 agonist that has a 97% homology to native GLP-1. The molecule has been modified to extend the circulating half-life from native GLP-1's 1–2 min to 13 h (Saxenda™ Product Label (FDA) 2021; Saxenda™ Product Label (EMA) 2021). Liraglutide reduces body weight through reduction of food intake (Holst 2007). Liraglutide is indicated for treatment of type 2 diabetes at a dose of up to 1.8 mg. The indication for chronic weight management is for liraglutide dosed at 3.0 mg, given once daily by injection. A dose escalation is required to minimize side effects, beginning at 0.6 mg and increasing by 0.6 mg weekly to the recommended dose of 3.0 mg daily (Saxenda™ Product Label (FDA) 2021; Saxenda™ Product Label (EMA) 2021).

Efficacy of Liraglutide Three 56-week studies with liraglutide 3.0 mg form the basis for regulatory approval (Pi-Sunyer et al. 2015; Wadden et al. 2013; Davies et al. 2015). One of those studies had an extended follow-up to determine the effect on emergence of type 2 diabetes in at-risk persons (Le Roux et al. 2016). In a large multi-center phase III trial called SCALE Obesity and Prediabetes, 3,731 patients without diabetes were instructed in a 500 kcal/day deficit diet and lifestyle recommendations and were treated in a ratio of 2:1 with liraglutide 3.0 mg/day (after dose titration) or with placebo (Pi-Sunyer et al. 2015). Liraglutide reduced body weight in those who completed 56 weeks by an average of 8.4 kg compared to 2.8 kg on average in the placebo-treated group. Weight loss of >5% was achieved by 62.3% of those receiving liraglutide but only 34.4% in those with placebo. The corresponding numbers losing >10% were 33.9% for those on liraglutide 3.0 mg and 15.4% for those assigned to placebo (Pi-Sunyer et al. 2015). The patients with prediabetes in the trial were followed out to 3 years to determine the effect on diabetes prevention (Le Roux et al. 2016). At 160 weeks, 26 of 1,472 individuals in the liraglutide 3.0 mg/day treatment group (2%) and 46 of 738 (6%) taking placebo were diagnosed with diabetes while on treatment. The time to onset of diabetes diagnosis with liraglutide 3.0 mg/day was 2.7 times longer than with placebo ($P < 0.0001$) (Le Roux et al. 2016).

Another trial, called SCALE Maintenance, had a unique design where weight loss of at least 5% was induced with a low-energy diet before patients were randomized to lifestyle counseling and either placebo or 3.0 mg/day (after titration) liraglutide (Wadden et al. 2013). This study is illustrated in Fig. 6, below. Weight loss on the highly structured low-calorie diet given for up to 12 weeks was 6% on average. After randomization, those receiving liraglutide 3.0 mg had additional loss of mean 6.2% (SD 7.3) and for placebo only 0.2% (SD 7.0). The percentage losing 5% and 10% of body weight was more than twice as high in the liraglutide treated patients (Wadden et al. 2013).

SCALE Diabetes trial (Davies 2015) illustrates not only the weight loss effect of liraglutide 3.0 mg, but also delineates the drug's effect on glycemia. In this study, patients with type 2 diabetes received a lifestyle intervention and were randomized to liraglutide 3.0 mg, liraglutide 1.8 mg, or placebo. At week 56, mean weight losses from baseline were, respectively, 6.0%, 4.7% and 2.0%. Exploratory comparisons of liraglutide 3.0 mg vs. 1.8 mg in this study showed that while weight loss differences were clinically and statistically superior for liraglutide 3.0 mg, the effect on glycemia, while statistically significant was small (−0.19%) (Davies et al. 2015).

Liraglutide has also been studied in patients with obstructive sleep apnea who could not tolerate conventional treatment with continuous positive airway pressure. In that study, (Blackman et al. 2016) the primary endpoint was reduction in apnea-hypopnea events per hour. There was a significant reduction in mean events when a lifestyle intervention was given with liraglutide 3.0 mg vs. placebo (−12.2 vs. −6.1 events per hour). In these patients, there were also improvements in body weight, systolic blood pressure, and Hemoglobin A_{1c} (Blackman et al. 2016).

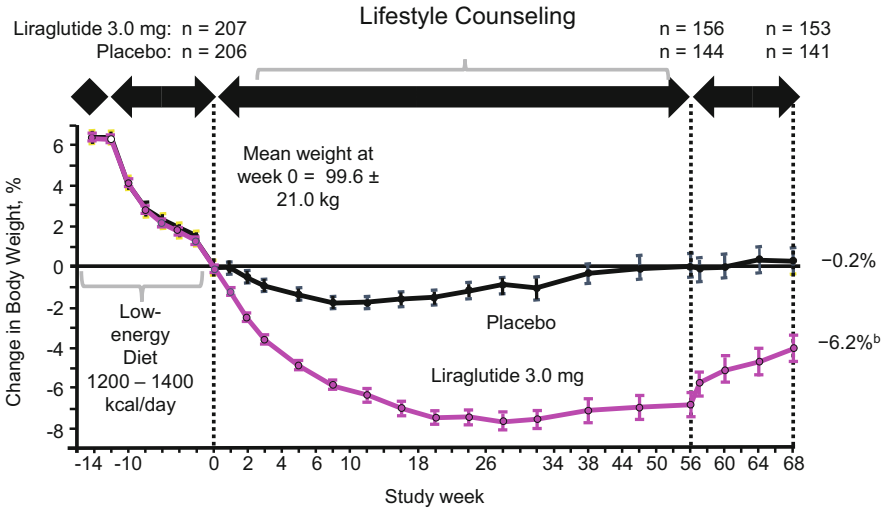


Fig. 6 SCALE Maintenance Study: Percent change in body weight over an initial period of highly structured low-calorie diet (1,200–1,400 kcal per day) is depicted from week 0 to −14. Participants who lost at least 5% received continued lifestyle counseling and were randomized to placebo or Liraglutide 3.0 mg. Percent change in body weight is depicted over 56 weeks for each treatment. After treatment stopped at week 56, participants returned for 4 follow-up visits through week 68. Note that after initial weight loss on diet alone, participants receiving lifestyle counseling and placebo maintained weight loss. Those on liraglutide 3.0 mg lost additional weight. After medication was stopped at week 56, weight regain is observed (image redrawn from Wadden et al. 2013). $P < 0.0001$ at week 56 for liraglutide vs. placebo

Safety Profile of Liraglutide As with other drugs in the GLP-1 receptor agonist class, Liraglutide is contraindicated in people with a family history of medullary thyroid carcinoma or multiple endocrine neoplasia syndrome type 2 (MEN2) (Saxenda™ Product Label (EMA) 2021; Saxenda™ Product Label (FDA) 2021). As with all medications for weight management, it is contraindicated in pregnancy. Liraglutide should not be studied in patients with a history of pancreatitis and should be discontinued if acute pancreatitis develops (Saxenda™ Product Label (EMA) 2021; Saxenda™ Product Label (FDA) 2021). Its safety when combined with other drugs for weight management has not been established. This drug is given by injection, and nausea was one of its most troublesome side effects, occurring in 39.3% of those on liraglutide compared to 13.8% in the placebo-treated group (Saxenda™ Product Label (EMA) 2021; Saxenda™ Product Label (FDA) 2021). Diarrhea, constipation, vomiting, dyspepsia, and abdominal pain also occurred in more than 5% of those treated with liraglutide (Saxenda™ Product Label (EMA) 2021; Saxenda™ Product Label (FDA) 2021). Mean serum calcitonin was statistically significantly higher in the liraglutide group but did not require further follow-up and calcitonin monitoring is not required (Saxenda™ Product Label (EMA) 2021; Saxenda™ Product Label (FDA) 2021). Hypoglycemia was only a problem in patients also taking sulfonylureas (Saxenda™ Product Label (EMA) 2021;

Saxenda™ Product Label (FDA) 2021). Blood pressure was significantly reduced, but pulse rate increased by an average of 2.5 beats/min. An increase of >10 beats/min was seen in 34% of the liraglutide treated group compared with 19% in the placebo-treated group (Saxenda™ Product Label (EMA) 2021; Saxenda™ Product Label (FDA) 2021). There were no changes in serum lipids. Liraglutide should be used with caution in patients with renal impairment (Saxenda™ Product Label (EMA) 2021; Saxenda™ Product Label (FDA) 2021). If weight loss does not exceed 4% by 16 weeks, the drug should be discontinued (Saxenda™ Product Label (EMA) 2021; Saxenda™ Product Label (FDA) 2021).

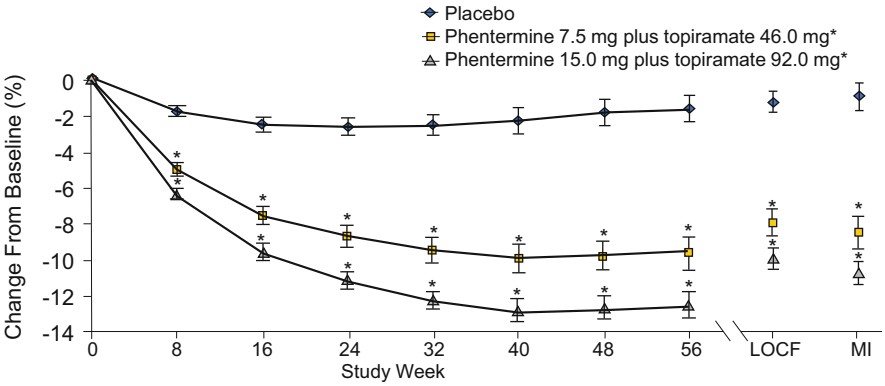
Liraglutide has been approved at a lower dose of 1.8 mg/day for the treatment of diabetes and is marketed under a different name. The indications for these two doses are distinct – if patients with and without diabetes are undertaking a weight loss effort, liraglutide 3.0 mg may be indicated but if the primary goal is management of glycemia in patients with diabetes, then liraglutide 1.8 mg is indicated. A cardiovascular outcome trial with liraglutide 1.8 mg/day has been completed (Marso et al. 2016). The endpoint was a combined index of major cardiovascular events which was reduced significantly in the patients receiving liraglutide, indicating clinical superiority for reduced CVD incidence over the placebo-treated group (Marso et al. 2016).

3.4 Phentermine/Topiramate Extended Release (ER) (Available in the USA, But Not Available in the EU)

The combination of phentermine and topiramate as an extended release (ER) form (PHEN/TPM ER) is approved for chronic weight management in the USA. It is not approved in the EU due to unresolved concerns on cardiovascular and psychiatric safety (Qsivia Assessment Report 2013). Phentermine acts to reduce appetite through increasing norepinephrine in the hypothalamus; topiramate may reduce appetite through its effect on GABA receptors (Qsymia™ Product Label, FDA 2021). The combination contains lower doses of phentermine (3.75–15 mg) than are usually prescribed when phentermine is used as a single agent. The dose of topiramate in the combination is between 23 and 92 mg, (Qsymia™ Product Label, FDA 2021) and is also lower than when topiramate is typically used for migraine prophylaxis or to control seizures.

Two clinical studies (Allison et al. 2012; Gadde et al. 2011) provided efficacy and safety data for approval of this medication (FDA 2021). The first trial, called EQUIP (Allison et al. 2012), enrolled subjects ≤70 years of age with BMI ≥35 kg/m^2 with controlled blood pressure (≤140/90 mmHg using 0–2 antihypertensive medications), fasting blood glucose ≤110 mg/dL, and triglycerides ≤200 mg/dL using 0 or 1 lipid lowering medication. EQUIP randomized participants to placebo or PHEN/TPM doses of 3.75/23, and 15/92 mg and achieved mean weight loss of 1.6%, 5.1%, and 10.9% of baseline body weight (Allison et al. 2012).

The other study, called CONQUER (Gadde et al. 2011), enrolled adults ≤70 years of age with BMI between 27 and ≤45 kg/m^2, except that patients with



Patients	Placebo	Mid	Full
Completers (% of randomized)	557 (57%)	338 (69%)	625 (64%)

Fig. 7 CONQUER study: percent change in body weight over 56 weeks with lifestyle intervention and randomization to placebo or phentermine 7.5 mg/topiramate 46 mg or phentermine 15 mg/topiramate 92 mg. The mean weight loss of last observation carried forward (LOCF) and multiple imputation(MI) data analysis is shown at week 56. Bars indicate standard errors. Figure redrawn from Gadde et al. (2011). *Weight change for either dose vs. placebo, $P < 0.0001$

type 2 diabetes had no lower BMI limit. The patients in the CONQUER study had 2 or more of the following comorbidities: hypertension, hypertriglyceridemia, dysglycemia (impaired fasting glucose, impaired glucose tolerance, or type 2 diabetes) or an elevated waist circumference (≥40 in. for men or ≥35 in. for women). At 56 weeks, body weight loss was least-squares mean 1.2% (95% CI 1.8 to 0.7) for placebo, 7.8% (CI 8.5 to 7.1; $P < 0{\cdot}0001$) for those on PHEN/TPM at the 7.5/43 mg dose and 10.2 kg (95% CI 10.4 to 9.3; $P < 0{\cdot}0001$) for PHEN/TPM at the 15/92 mg dose (Gadde et al. 2011). The CONQUER study results are depicted in Fig. 7.

The patient population in the EQUIP and CONQUER studies represents those with higher risk profiles from the consequences of excess weight. A titration period of 2 weeks is required for PHEN/TPM ER, starting at 3.75/23 mg dosage. This combination medication produces mean weight losses approaching 10% more than placebo which is larger than observed in clinical trials with single drugs (Colman et al. 2012).

The SEQUEL study (Garvey et al. 2012) was a second-year extension of the CONQUER study keeping those patients who participated in their initial treatment assignment (SEQUEL). Patients completing 2 years at the dose of 7.5 mg/46 mg maintained a mean weight loss of 9.3% below baseline and those on the top dose maintained a mean 10.7% weight loss from baseline (Garvey et al. 2012).

Improvements in blood pressure, glycemic measures, HDL cholesterol, and triglycerides occurred with both the recommended and the top doses of the medication in these trials (Qsymia product label, FDA). Improvements in risk factors were related to the amount of weight loss (Allison et al. 2012; Gadde et al. 2011). In

patients with sleep apnea this combination reduced the severity of symptoms (Garvey et al. 2012).

The most observed side effects in these clinical trials were paresthesia, dizziness, dysgeusia (altered taste), insomnia, constipation, and dry mouth (Qsymia™ Product Label, FDA 2021). These side effects are related to the constituents of PHEN/TPM ER or, in the case of constipation, to weight loss per se. Phentermine causes insomnia and dry mouth, usually early in treatment, which then resolves. Topiramate is a carbonic anhydrase inhibitor that is associated with altered taste for carbonated beverages and tingling in fingers, toes, and perioral areas and may lead to mild metabolic acidosis.

Safety concerns are seen in several areas. This drug is contraindicated in pregnancy, as are all weight loss medications, but the topiramate constituent requires special precautions in women of childbearing potential (Qsymia™ Product Label, FDA 2021). If a patient becomes pregnant while taking PHEN/TPM ER, treatment should be stopped immediately (Qsymia™ Product Label, FDA 2021). Topiramate is associated with oral clefts if used during early pregnancy and PHEN/TPM ER is thus US pregnancy Category X (Qsymia™ Product Label, FDA 2021). Because of the risk of oral clefts, a negative pregnancy test before treatment and monthly thereafter and use of effective contraception are required (Qsymia™ Product Label, FDA 2021). Glaucoma is a rare side effect of topiramate, and the drug is contraindicated in glaucoma (Qsymia™ Product Label, FDA 2021). PHEN/TPM ER is also contraindicated in hyperthyroidism and within 14 days of treatment with monoamine oxidase inhibitors (MAOIs) and in patients with hypersensitivity to any of the ingredients in the medication (Qsymia™ Product Label, FDA 2021). Other potential issues include risk of kidney stones (associated with topiramate) and increased heart rate in patients susceptible to phentermine (Qsymia™ Product Label, FDA 2021).

4 Obesity Pharmacotherapy for the Next Decade

Semaglutide has shown clinicians how to significantly affect energy balance by affecting appetite (Friedrichsen et al. 2021). And setmelanotide is a great example of personalizing obesity therapy, albeit with a challenge of identifying a broader population that might benefit from the drug, beyond the ultra-rare genetic and syndromic obesities. Tirzepatide, now in phase 3, and bimagrumab, in phase 2, are illustrative of two different approaches that might make an impact on clinical practice in the next decade.

4.1 Tirzepatide

Tirzepatide, a single-molecule with a dual-action, given as once-weekly injection, targets both the glucagon-like peptide-1 (GLP-1) receptor and the glucose-insulin peptide (GIP) receptor. In a phase 2 trial it produced mean weight loss in the range of

~12% at 26 weeks at a dose of 15 mg/day and had potent effects on glycemia (Frias 2008). Tirzepatide is being evaluated for obesity in SURMOUNT-1, a phase 3 randomized double-blind, placebo-controlled trial with 2,400 participants who have obesity and comorbidity, but not diabetes (ClinicalTrial.gov). The drug is also being evaluated for an indication for type 2 diabetes in a series of studies, SURPASS (Min and Bain 2021). The results of one of the phase 3 studies has been released publicly, but not yet published in a peer-reviewed format. In that study, the highest dose (15 mg) of tirzepatide produced 13.1% weight loss over 40 weeks in persons with type 2 diabetes (Lilly News Release 2021). The safety and efficacy of tirzepatide in persons with obesity will be watched closely. The combined targeting of GLP-1 and GIP is interesting, and it will be important to understand the mechanistic pathway by which tirzepatide produces weight loss – appetite, lipolysis, and energy expenditure effects should all be investigated.

4.2 Bimagrumab

Bimagrumab is a human monoclonal antibody that binds to the activin type II receptor (ActRII) to block natural ligands that negatively regulate skeletal muscle growth (Rooks et al. 2017; Heymsfield et al. 2021). Bimagrumab was tested in a double-blind, placebo-controlled, 48-week, phase 2 randomized clinical trial (Heymsfield et al. 2021) in adults with type 2 diabetes and body mass index $28 \leq 40$ kg/m^2. Bimagrumab or placebo was dosed at 10 mg/kg up to 1,200 mg in 5% dextrose solution every 4 weeks for 48 weeks; both groups received diet and with both DEXA and MRI being used for body composition. At week 48, the changes for bimagrumab vs. placebo were as follows: fat mass (FM), −20.5% (−7.5 kg [80% CI, −8.3 to −6.6 kg]) vs. −0.5% (−0.18 kg [80% CI, −0.99 to 0.63 kg]) ($P < 0.001$); lean mass (LM), 3.6% (1.70 kg [80% CI, 1.1 to 2.3 kg]) vs. −0.8% (−0.4 kg [80% CI, −1.0 to 0.1 kg]) ($P < 0.001$) (Heymsfield et al. 2021). Thus rather than loss of both lean and fat with weight loss with the typical ratio of 25:75 (Heymsfield et al. 2014), bimagrumab was associated with loss of fat mass and gain in lean mass (Heymsfield et al. 2021). Safety will need to be evaluated further; there were cases of elevations of pancreas and liver enzymes with bimagrumab compared to placebo in this small study (Heymsfield et al. 2021).

4.3 Cagrilintide + Semaglutide

Cagrilintide is a long-acting amylin analog. It is being developed as a combination approach with semaglutide. It was evaluated in a phase 1b study and semaglutide 2.4 mg + 2.4 mg or 4.5 mg cagrilintide produced weight loss at 20 weeks that was −17.1% and 15.1% in those two doses (Enebo et al. 2021). The molecule has been studied in phase 2 for obesity treatment (Fletcher et al. 2021). The combination's robust early weight loss shows promise for even greater long-term weight loss (Becerril and Frühbeck 2021). A study found on ClinicalTrials.gov documents a

study comparing the two drugs injected separately or as two injections. The combination is not yet in phase 3, however.

5 The Way Forward in Obesity Pharmacotherapy

There are other drugs in the pipeline that show various degrees of promise and the reader is referred to recent reviews for addition information on individual drugs (Srivastava and Apovian 2018; Rebello and Greenway 2020). Rather than singling out individual agents, a few comments on the path forward are in order. We need more drugs that work through appetite, like semaglutide does in targeting the GLP-1 receptors in the areas of the brain that affect appetite. Not all patients respond to semaglutide with enough weight loss; not all patients can tolerate semaglutide; additional medications are needed. We need more medications that take a personalized approach, like setmelanotide. With better phenotyping and better genotyping, we should be better able to develop targeted therapies for individuals based on the personal profile of the patient with obesity. We need to consider mechanisms of promoting negative energy balance other than reducing food intake through appetite effects. One positive aspect of setmelanotide is that it increases energy expenditure, an important quality in the face of the metabolic adaptation found with the weight reduced state. Setmelanotide appears to do this without cardiovascular effects of increased blood pressure and pulse. Tirzepatide offers the intriguing possibility that its effectiveness in weight loss may be more than just food intake. Increasing lipolysis is a viable hypothetical mechanism for one of this drug's mechanism of action. Bimagrumab gives the first evidence that we might succeed in targeting improved quality of weight loss for our patients. We might be able to preserve or even increase lean mass, especially muscle and bone, in our patients as they lose weight.

The goal of weight loss is health improvement. Obesity medicine specialists want to reduce the excess abnormal adipose tissue that is driving ill health. At the same time, we want to achieve healthy weight reduction with preservation of muscle and bone. Can we achieve these goals pharmacologically? Of course, it would be better to live in a world where healthy eating and active living were the default behaviors and where those behaviors were reinforced in a world without undue emotional and financial stress. Those social determinants drive risk for obesity. All of us need to work toward creating that world, but we also need to explore better pharmacologic options for weight management for those who need to lose weight as a pathway to better health. The next generation of anti-obesity medications is emerging, bringing the possibility of weight loss sufficient to produce meaningful health improvement in many patients with obesity. But we need to continue the efforts to identify other medications and to shift our focus to more than just weight loss. We need to start thinking about improved quality of weight loss.

The clinical practice of obesity medicine has until now been a struggle for patients and providers. At last, we are getting some powerful tools to help our patients. The focus can finally shift from treating all the complications of obesity

with antihypertensives, with lipid lowering drugs, with glycemia management drugs. We can finally focus on the root cause of these comorbidities – obesity – because we can finally do something about it.

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Author Contribution: I conceived and created this document in its entirety.

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Novel Drugs for Diabetes Therapy

Tim Heise

Contents

Abstract

Since the first use of insulin 100 years ago, there have been marked improvements in diabetes therapy including, but not limited to, the development of oral antidiabetic agents (OADs), incretin mimetics and insulin analogues. Still, there are substantial shortcomings in diabetes therapy: the blood-glucose lowering effect of OADs is often limited, incretin mimetics often induce gastrointestinal

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side effects and insulins still induce hypoglycaemia and weight gain in many patients.

This review evaluates on-going developments of antidiabetic drugs for their potential for future therapy focussing on injectable therapies. Recent data from dual agonists, in particular tirzepatide, a combination of GIP- and GLP-1 receptor agonists, show unprecedented reductions in HbA1c, body weight and cardiovascular risk factors. Once-weekly administrations of incretin mimetics open up the potential of a combination with once-weekly insulins that have been shown to have low peak-to-trough fluctuations. Eventually, it might be feasible to administer incretins and insulins (combinations) orally. While this has already been achieved for incretins, there are still some challenges for the oral application of insulin. Nevertheless, many promising data of novel antidiabetic drugs clearly indicate that therapy of people with diabetes will become easier, safer and more efficacious in the next years.

Keywords

Diabetes · Dual agonists · GLP-1 receptor agonist · Incretin · Insulin · Therapy

1 Introduction

Pharmacological diabetes therapy started with the first use of insulin 100 years ago. Since then, there have been quite a number of additions to the therapeutic armamentarium, such as oral antidiabetic agents (OADs) and incretin mimetics. Notably, SGLT-2 inhibitors and GLP-1 receptor agonists have been shown to reduce the excess cardiovascular risk of people with diabetes compared to non-diabetic people (Buse et al. 2020). The insulin molecule itself and insulin formulations have also been optimised for therapeutic use, e.g., by purification of insulin formulations reducing antibody formation, by adding mechanisms to slow down insulin absorption for basal insulin coverage and by modifications to accelerate insulin absorption for prandial insulin therapy (Home 2021; Heise 2021a).

Nevertheless, there are still major shortcomings in diabetes therapy. OADs often have limited blood-glucose lowering properties or are associated with side effects including hypoglycaemia and body weight gain. The therapeutic potential of incretin mimetics can often not fully exploited because of gastrointestinal side effects (Hayes et al. 2021). Despite all refinements, insulin formulations are still associated with the highest risk of hypoglycaemia among all available antidiabetic therapies, in particular in people with type 1 diabetes and long-standing type 2 diabetes (UK Hypoglycaemia Study Group 2007). In addition, the rather inconvenient subcutaneous route of administration (which also applies to most incretin mimetics) in combination with adverse effects like body weight gain and other psychosocial factors makes many patients and clinicians reluctant to initiate insulin therapy (Khunti and Millar-Jones 2017).

Looking at the growing number of patients with diabetes and the still unsatisfactory control in many patients (Kazemian et al. 2019), there is a clear unmet need for novel drugs facilitating and improving diabetes therapy. This review will describe some on-going developments of blood-glucose lowering drugs focussing on injectables. Indeed, there are a number of promising developments on-going for incretin mimetics with even higher efficacy, in particular dual combinations of either glucose-dependent insulin releasing polypeptide (GIP) or glucagon receptor agonists and glucagon-like peptide-1 (GLP-1) mimetics. Likewise, first data have been provided for improved insulin formulations, making insulin initiation easier and reducing undesired side effects of insulin therapy. These include but are not limited to insulin for once-weekly application and orally administered insulins. These two insulin developments seem to be the most promising, not only because they have already entered at least phase 2 of the clinical development stage, but also because they could possibly be combined with incretin mimetics or incretin combinations.

In contrast, other insulin developments such as hepato-preferential insulin primarily acting on the liver or glucose-responsive insulins that only act when blood glucose levels are high are still in early development stage with only few clinical data available that mostly show the high challenges that have to still to be overcome for these developments (Heise 2021a).

Likewise, there do not seem to be too many oral agents in late-stage development that might enter the diabetes market soon (Bailey and Day 2018). Small molecule agonists of G-protein-coupled fatty acid receptors GPR40 and GPR119 have been shown to increase insulin secretion or decrease glucagon secretion, but have not entered late-stage clinical developments yet or have been discontinued due to hepatic side effects (Bailey and Day 2018). Glucokinase activators are very potent insulin secretion enhancers that, however, often lead to hypoglycaemia and also show tachyphylaxis (Nakamura and Terauchi 2015). The development of other oral drug candidates, such as inhibitors of the 11ß-hydroxysteroid-dehydrogenase, were stopped because of lack of efficacy (Bailey and Day 2018; Bianzano et al. 2021).

Currently, the only OAD in late-stage development seems to be imeglimin, a tetrahydrotriazine-containing compound that improves both insulin secretion and insulin action due to modulation of mitochondrial function according to a recent review co-authored by staff of Poxel, the manufacturer of imeglimin (Hallakou-Bozec et al. 2021). Imeglimin has been approved in Japan, but not yet in the EU or the USA. Phase 2b and phase 3 clinical trials with 74–107 patients per arm showed good tolerability and an HbA1c-lowering effect of about 0.9% with 1,000 mg imeglimin versus placebo (Dubourg et al. 2021a, b). There were no differences with regard to body weight. Full publications with active comparators have not yet been provided. Most importantly, there are no cardiovascular outcome data available for imeglimin, and it has to be applied twice daily in contrast to SGLT-2 inhibitors that are mostly applied once daily. Due to these limitations, a wide-spread use of imeglimin seems unlikely in view of the competition with SGLT-2 inhibitors with proven cardiovascular benefits.

2 GLP-1 Receptor Co-agonists

2.1 Background

Undoubtedly, GLP-1 mimetics have substantially changed diabetes therapy. Robust improvements in both glycaemic control and body weight in combination with proven cardiovascular benefits have moved GLP-1 mimetics to the first-line injectable antidiabetic therapy (Buse et al. 2020). Moreover, the availability of the first GLP-1 mimetic for oral administration might further increase the popularity of this class of compounds.

However, GLP-1 mimetics still have some limitations. Because of rather frequent gastrointestinal side effects, the dose has to be up-titrated over weeks to achieve the maximum tolerated dose. But even at this dose level GLP-1 mimetics do not yet achieve the efficacy of bariatric surgery for body weight reduction and remission of type 2 diabetes (Baggio and Drucker 2021). In physiology, most peptide-secreting cells in the gut are plurihormonal, so that the gut secretes several peptide hormones in addition to GLP-1 to control body weight and glucose levels (Drucker 2016). It might therefore make sense to look into combinations of GLP-1 (mimetics) with other gut-secreted hormones like glucagon and GIP. Indeed, both pre-clinical studies and data from patients after bariatric surgery showing sustained increases in the circulating levels of multiple gut hormones indicate a high therapeutic potential of such combinations (Baggio and Drucker 2021; Finan et al. 2015).

2.2 Combinations of GLP-1 Agonists and Glucagon

While glucagon is usually regarded as a hormone with insulin-counterregulatory action increasing glucose levels, it also inhibits food intake and induces weight loss similar to the actions of GLP-1 (Henderson et al. 2016). Also, the naturally occurring 37 amino acid peptide oxyntomodulin contains the amino acid sequence of glucagon (GCG) and an 8 amino acid carboxy terminal extension and binds to the glucagon and GLP-1 receptors (Baggio and Drucker 2021). It has been shown that the weight-sparing effect of oxyntomodulin is mediated by both the glucagon and GLP-1 receptor (Kim et al. 2018a) inducing appetite suppression and satiety, but also increasing energy expenditure (Wynne et al. 2006). Therefore, a glucagon/GLP-1 co-agonist is an attractive candidate for treatment of diabetes and obesity (Baggio and Drucker 2021). Indeed, several of such co-agonists have been investigated, at least pre-clinically, however, the optimal ratio of glucagon and GLP-1 receptor activation (i.e., the affinity to each receptor) has not been easy to find.

While there are a number of glucagon/GLP-1 co-agonists in development (Hope et al. 2021), the most promising data seem to come from cotadutide which shows a balanced activity towards the glucagon and GLP-1 receptors (Ambery et al. 2018). In a placebo-controlled study in people with type 2 diabetes cotadutide showed a placebo-corrected decrease in postprandial glucose levels of 22% and reduced body weight by more than 2 kg in 41 treatment days (Ambery et al. 2018). In addition, a

subset of patients was investigated with MRI-based proton-density fat-fraction analysis and showed a significantly stronger decline from baseline in liver fat (−6.0% vs. −3.2%), subcutaneous adipose tissue and visceral adipose tissue ($p = 0.052$) with cotadutide than with placebo.

The glucose-lowering effects of cotadutide were confirmed in a 4-week treatment study in overweight subjects with type 2 diabetes treated with dapagliflozin and metformin. Cotadutide showed significantly larger improvements versus placebo in both postprandial glucose levels (a difference of 22%) and mean glucose levels assessed by CGM (difference of 34 mg/dl) (Flor et al. 2021). The glucose-lowering properties of cotadutide seem to be primarily mediated by enhanced insulin secretion and delayed gastric emptying (Parker et al. 2020). Pre-clinical studies indicated that the effects of cotadutide to reduce body weight, food intake and improve glucose control are predominantly mediated through the GLP-1 signalling (Boland et al. 2020). In contrast, its action on the liver to reduce lipid content is directly mediated through glucagon signalling (Boland et al. 2020).

In a phase 2b-study in patients with type 2 diabetes inadequately controlled with metformin, cotadutide (investigated in doses of 100 μg, 200 μg and 300 μg q.d.) again led to significant reductions in body weight and HbA1c versus placebo over a treatment duration of 54 weeks (Nahra et al. 2021). There were no significant differences in HbA1c or body weight between the cotadutide arms versus liraglutide 1.8 mg q.d. with the exception of a slightly, but significantly higher body weight loss in the 300 μg cotadutide arm (−5.01 kg vs. −3.44 kg with liraglutide, $p = 0.001$). Patients in this treatment arm also showed greater reductions in liver enzymes, i.e. ALT ($p = 0.023$), whereas lower doses only showed significant reductions in liver enzymes (AST, ALT and GGT) versus placebo. However, the results of this study are difficult to interpret as cotadutide treatment was associated with significantly higher rates of treatment-emergent adverse events (TEAEs) across all doses. The most commonly reported TEAEs with cotadutide treatment were gastrointestinal disorders, including diarrhoea, nausea and vomiting. While a marked decrease in the event rate of these gastrointestinal events was observed over time, cotadutide showed lower study completion rates compared with placebo or liraglutide (73–77% cotadutide, 94% liraglutide, 81% placebo). Additional data are therefore needed to evaluate cotadutide's tolerability and its efficacy in comparison with more potent glucose-lowering therapies than liraglutide (e.g., semaglutide).

2.3 Combinations of GLP-1 Agonists and GIP

The most prominent candidate of the combination of a GLP-1 mimetic and GIP is tirzepatide for which the phase 3 programme has already been completed. In contrast, most other GIP/GLP-1 co-agonists are still in pre-clinical development (Burade et al. 2021), however, some clinical data were published for an acylated GLP-1/GIP co-agonist demonstrating good tolerability at doses of up to 3.6 mg in healthy people and people with type 2 diabetes (Portron et al. 2017; Schmitt et al. 2017) as well as significant reductions in HbA1c and body weight over 12 weeks in

people with type 2 diabetes (Frias et al. 2017). However, these improvements (in contrast to those seen with tirzepatide) did not exceed those observed with the GLP-1 agonist liraglutide alone.

Tirzepatide is a 39 amino acid linear peptide attached to a C20 fatty diacid moiety allowing binding to albumin thereby achieving a mean half-life of ~5 days (116.7 h), enabling once-weekly dosing (Coskun et al. 2018). Its molecular structure is based on the native GIP peptide sequence, but was modified to bind to both GIP and GLP-1 receptors. In vitro, tirzepatide has a higher potency to native GIP and has an about 5 times lower affinity to the GLP-1 receptor than native GLP-1 (Coskun et al. 2018; Papachristou et al. 2021). The higher affinity to the GIP-receptor might be surprising as even very high doses of GIP were ineffective as an insulinotropic agent in subjects with type 2 diabetes. In addition, GIP receptor knockout mice were protected from weight gain induced by high fat-feeding, suggesting that GIP might induce rather than prevent weight gain (Nauck et al. 2021).

Still, GIP is an attractive candidate for the combination with GLP-1 as both incretins have important physiological effects that are only partly overlapping. GLP-1 improves glucose-dependent insulin secretion, reduces glucagon secretion, slows down gastric emptying and leads to weight loss mainly through reducing appetite (Nauck and Meier 2016). GIP has also been demonstrated to have glucose-dependent effects on insulin and glucagon secretion, with a potentiation of glucagon secretion at hypoglycaemic and euglycaemic conditions but no effect on glucagon secretion at hyperglycaemic conditions where it potentiates the glucose-induced insulin secretion (Christensen et al. 2011). GIP was shown to increase glucose uptake, but also to promote the activity of lipoprotein lipase and of lipogenesis leading to increased triglyceride storage in adipose tissue (Nauck et al. 2021). In addition, GIP is involved in meal-associated bone remodelling (Nauck et al. 2021).

Overall, the effects of GIP alone are difficult to study in humans because of its short half-life. While a long-acting GIP receptor agonist has been developed for rodent studies (Zhang et al. 2021), such a tool is not available for human research. Potential benefits of the combination of GLP-1 and GIP can therefore only be derived from studies with co-agonists such as tirzepatide. Tirzepatide has been shown to suppress fasting glucagon more than the GLP-1 receptor agonist dulaglutide, even after adjusting for ambient plasma glucose concentrations (Nauck et al. 2021). In addition, it was recently demonstrated in a glucose clamp study that tirzepatide improves both insulin secretion and insulin sensitivity to a significantly larger extent than does the GLP-1 receptor agonist semaglutide (Heise 2021b). While these studies clearly indicate a high therapeutic potential for GLP-1/GIP-receptor co-agonists in diabetes and obesity, they cannot answer the question how and to what extent GIP contributes to the overall effects of these co-agonists. Based on pre-clinical findings (Borner et al. 2021) it has also been proposed that GIP acts as an anti-emetic, thereby improving the tolerability of GLP-1 receptor agonists (Hayes et al. 2021). Indeed, gastrointestinal side effects limit the efficacy of GLP-1 receptor agonists and it has been suggested that more than 20% of patients with type 2 diabetes cannot benefit fully from existing GLP-1 therapeutics because of these adverse events (Hayes et al. 2021). Improving gastrointestinal tolerability may

Table 1 Summary of the SURPASS phase 3 programme

Trial	Patient population	Comparator	Tirzepatide doses	Treatment duration	Blinding
SURPASS-1 (Rosenstock et al. 2021)	T2DM, diet/exercise	Placebo	5, 10, 15 mg	40 weeks	Double-blind
SURPASS-2 (Frias et al. 2021a)	T2DM, metformin monotherapy	Semaglutide	5, 10, 15 mg	40 weeks	Open-label
SURPASS-3 (Ludvik et al. 2021)	T2DM, metformin±SGLT-2 inhibitors	Insulin degludec	5, 10, 15 mg	52 weeks	Open-label
SURPASS-4 (Del Prato et al. 2021)	T2DM, 1–3 OADs (metformin, SGLT-2 inhibitor, SUs)	Insulin glargine	5, 10, 15 mg	52 weeks	Open-label
SURPASS-5 (Dahl et al. 2021)	T2DM, insulin glargine±metformin	Placebo	5, 10, 15 mg	40 weeks	Double-blind
SURPASS-6	T2DM, insulin glargine±metformin	Insulin lispro	5, 10, 15 mg	52 weeks	Open-label
SURPASS-CVOT	T2DM, confirmed atherosclerotic CVD	Dulaglutide	Not reported	Maximum 54 months	Double-blind

T2DM type 2 diabetes mellitus, *OADs* oral antidiabetic drugs, *SUs* sulfonylureas, *CVD* cardiovascular disease

SURPASS-6 and SURPASS-CVOT have not been published yet. Information can be found at https://clinicaltrials.gov/ct2/show/NCT04537923 and https://clinicaltrials.gov/ct2/show/NCT04255433 (access of 14 Nov 2021, respectively)

therefore allow for using higher doses of GLP-1 receptor agonists to further enhance weight loss and glycaemic control.

While the exact contribution of GIP is still unclear, there is no question that tirzepatide shows unprecedented effects on glycaemia and body weight. This was impressively shown in the SURPASS-studies, the phase 3 programme of tirzepatide (Rosenstock et al. 2021; Frias et al. 2021a; Ludvik et al. 2021; Del Prato et al. 2021; Dahl et al. 2021). Design and results of these studies are summarised in Table 1 and Fig. 1. Overall, tirzepatide was investigated in people with type 2 diabetes in different treatment modalities (treatment-naïve, metformin monotherapy or in combination with other oral antidiabetic drugs, basal insulin therapy) and compared to placebo, semaglutide, insulin degludec or insulin glargine over 40 or 52 weeks. Tirzepatide was administered at once-weekly doses of 5 mg, 10 mg and 15 mg. To improve tolerability, tirzepatide treatment was initiated at 2.5 mg once per week, and increased by 2.5 mg every 4 weeks until the randomised dose was achieved. In case of intolerable gastrointestinal adverse events, the tirzepatide dose could be reduced to a lower, tolerated maintenance dose on which participants remained for the remainder of the study. Dose de-escalation was allowed only once. Despite this up-titration schedule, gastrointestinal adverse events were rather high with tirzepatide, in particular at the start of treatment. Up to 26.4% of patients exposed

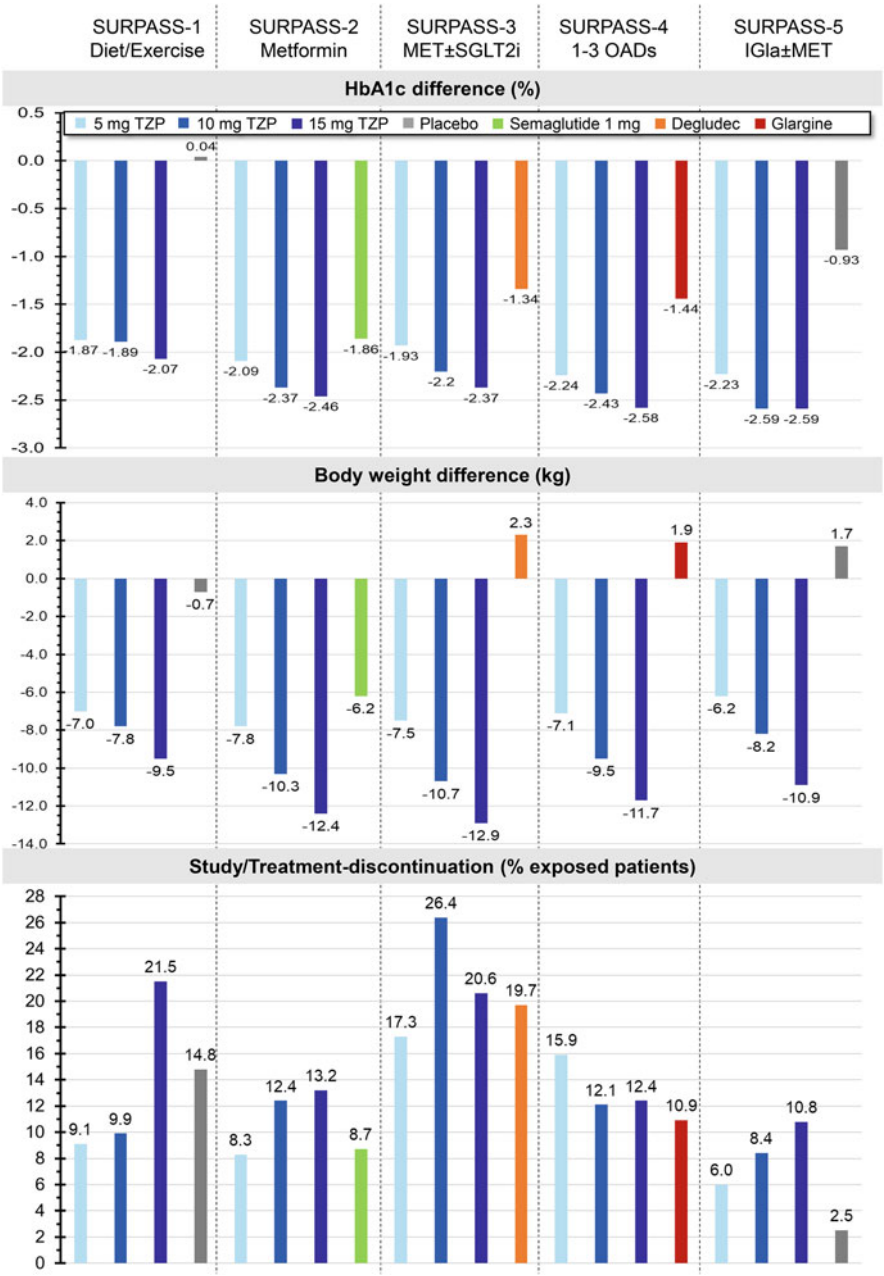

Fig. 1 Results of the Tirzepatide SURPASS Phase 3 Programme. The figure shows mean changes from baseline in HbA1c (upper panel) and body weight (middle panel) for the different tirzepatide doses and the comparators. The lower panel shows the percentage of randomised patients not completing the study or the full treatment duration. *MET* metformin, *SGLT-2i* SGLT-2 inhibitor, *OADs* oral antidiabetic drugs, *TZP* tirzepatide

to tirzepatide discontinued study participation or treatment (Fig. 1, lower panel), although not all these discontinuations were due to adverse events. Nevertheless, study or treatment discontinuation was often higher with tirzepatide than with comparators, often even in the 5 mg once-weekly study arm.

On the other hand, tirzepatide showed impressive reductions in HbA1c and body weight (Fig. 1, upper and middle panel) which were significantly higher than active comparators or placebo. Importantly, treatment effects were significantly higher than those achieved with semaglutide (Frias et al. 2021a) which in previous studies had shown superior effects versus other GLP-1 receptor agonists (Capehorn et al. 2020; Pratley et al. 2018). Up to 87% of patients achieved HbA1c-levels $\leq$6.5% and up to 51% even showed normal HbA1c-levels below 5.7% (Frias et al. 2021a). Body weight losses were also impressive with up to 43% of patients achieving a weight loss of at least 15% (Ludvik et al. 2021). In a CGM-substudy of SURPASS-3, patients on 10 mg or 15 mg Tirzepatide once weekly showed 91% of CGM-values to be in target range (70–180 mg/dl) with only 1% or less values in the hypoglycaemic range (Battelino et al. 2021). Hypoglycaemia did not substantially increase in the combination with insulin glargine with annual rates of level 2 hypoglycaemia (blood glucose $<$54 mg/dl) of 0.43–0.50 events versus 0.44 events with insulin glargine alone (Dahl et al. 2021). Furthermore, Tirzepatide reduced liver enzymes (ALT and AST) by up to 32% of baseline values and liver fat content by 8.1% versus 3.4% with degludec (absolute changes from baseline) (Gastaldelli et al. 2021). Abdominal visceral adipose tissue was lowered by up to 1.65 l from baseline compared to a subtle increase (0.38 l) with insulin degludec.

Overall, these results clearly indicate that tirzepatide and potentially other GLP-1/GIP receptor co-agonists will achieve major improvement in patients with type 2 diabetes, at least in those who can tolerate at least weekly doses of 5 mg or 10 mg. Data on the impact of tirzepatide on hard cardiovascular endpoints are still pending (a cardiovascular outcome trial has been started, https://clinicaltrials.gov/ct2/show/NCT04255433, access 4th Jan 2022). However, a post-hoc analysis of the phase 2 data with tirzepatide showed improvements in cardiovascular biomarkers of insulin resistance, inflammation, endothelial dysfunction and cellular stress biomarkers (Wilson et al. 2020, 2021). Nevertheless, the results of the on-going cardiovascular outcome trial are certainly will have to be awaited before being able to evaluate the potential cardiovascular benefit of tirzepatide, in particular as it (like GLP-1 receptor agonists alone) significantly increases heart rate, but lowers systolic and diastolic blood pressure (Del Prato et al. 2021).

2.4 Other Dual- or Triple Agonists with GLP-1

Other co-agonists with GLP-1 receptor agonists are still early in development and are discussed in excellent previous reviews (Baggio and Drucker 2021). One promising development is the combination of amylin and GLP-1 receptor agonists. Amylin acts as both a neuropeptide and circulating endocrine hormone co-secreted with insulin from islet beta-cells and seems to be involved in the control of energy

homeostasis, encompassing locomotor activity, energy expenditure and food intake (Baggio and Drucker 2021). Co-administration of a long-acting amylin analogue and semaglutide led to greater body weight reductions (15.7–17.1%) than semaglutide alone (9.8%) in a multiple-ascending dose, phase 1b trial in healthy overweight or obese people (Enebo et al. 2021). Large and long-term clinical trials including people with type 2 diabetes are outstanding.

Further promising candidates for the combination with GLP-1 receptor agonists are fibroblast growth factors (FGFs) as they decrease food intake, enhance thermogenesis, increase hepatic glucose uptake, reduce hepatic fat accumulation and liver inflammation and also enhance insulin action (Baggio and Drucker 2021). In pre-clinical studies, even single administrations of FGF1 (peripherally or into the brain) led to remissions of experimental diabetes (Bentsen et al. 2020). Clinical studies with analogues of FGF19 or FGF 21 showed some promising results such as reduction in liver fat and body weight, but not necessarily in blood glucose levels (Gaich et al. 2013; Harrison et al. 2018). Co-agonists of GLP-1 and FGF21 receptor agonists have only been studied pre-clinically so far. A fusion protein of the two agonists improved body weight and glucose control to a greater extent than the single components without inducing hypoglycaemia in rodents (Gilroy et al. 2020).

Finally, triple co-agonists have been investigated, although still pre-clinically. In view of the impressive efficacy of tirzepatide, GLP-1/GIP/glucagon triple agonists might be of particular interest. One of these triple agonists, HM15211, showed potent weight loss and HbA1c-improvements in obese animal models, either alone or in combination with a once-weekly insulin (Kim et al. 2018b). In addition, HM15211 reduced both body weight and liver fat content in obese subjects with non-alcoholic fatty liver disease (Abdelmalek et al. 2020).

Another GLP-1/GIP/glucagon triple agonist in development, LY3437943, has some similarities to tirzepatide as it is a single peptide derived from a GIP backbone and has an activity ratio between the GIP-receptor and GLP-1 receptor alike tirzepatide (Coskun et al. 2021). The glucagon receptor activity is similar to that of native glucagon. LY3437943 led to a significantly greater weight loss than cotadutide, liraglutide and tirzepatide, stimulated glucose-dependent insulin secretion and improved insulin sensitivity in DIO male C57/Bl6 mice (Coskun et al. 2021). It also reduced liver triglycerides and liver enzymes (ALT) in doses of 1 nmol/kg or higher in obese mice. A first in human study in healthy people showed good tolerability and safety, although (as expected) gastrointestinal adverse events were more frequent with LY3437943 (Urva et al. 2021). While no changes in fasting plasma glucose levels were observed in this single dose study, there were significant reductions in body weight with higher doses of LY3437943 (4.5 and 6.0 mg) which were sustained over the 43 days observation period and were most likely due to appetite suppression (Urva et al. 2021).

In summary, GLP-1 dual and triple receptor agonists show very promising results, in particular with regard to improvements in glucose control and body weight. While many of these agonists are still early in development, the GLP-1/GIP co-agonist tirzepatide has already completed phase 3 and is expected to get regulatory approval in the USA and Europe in 2022.

3 Novel Insulin Preparations

3.1 Once-Weekly Insulins

Obviously, one advantage of insulins that can be applied once weekly is the reduction in the number of necessary injections. Indeed, patients' fear of needles or social embarrassment associated with injecting in public is a major reason for omitting insulin doses or for initiating insulin therapy with negative implications for glycaemic control (Ross 2013). But the potential of once-weekly insulins goes beyond reducing needle anxiety or mere convenience. Because of their long half-life once-weekly insulins should have low peak-to-trough fluctuations, thereby potentially avoiding hypoglycaemia and/or improving glycaemic control (Heise and Meneghini 2014). On the other hand, short-term adaptations of insulin dose will not be possible with very long-acting insulins which may trigger concerns about potential periods of over- or under-insulinisation with high risks of hypoglycaemia or hyperglycaemia (Heise 2021a).

3.1.1 Insulin Icodec

To date, clinical data of two once-weekly insulins in development have been published. One is insulin icodec which differs from the human insulin molecule in three positions (A14E, B16H and B25H) and contains a C20 fatty diacid containing side chain at B29K which is attached via a hydrophilic linker (Nishimura et al. 2020). These modifications lead to strong, but reversible albumin binding (10 times stronger than the albumin binding properties of insulin detemir), reduced enzymatic degradation and attenuated insulin receptor binding and clearance. Once insulin icodec binds to the insulin receptor, it elicits the same metabolic effects as human insulin. The in vitro mitogenic effects of icodec were found to be lower than that of human insulin (Nishimura et al. 2020).

3.1.2 Basal Insulin Fc (BIF)

The other development is Basal Insulin Fc (BIF), a fusion protein that combines a novel single-chain variant of insulin with a human IgG_2Fc domain (Heise et al. 2021). BIF exhibits reduced insulin receptor (IR) potency with full agonism, and selectivity against human insulin-like growth factor-1 receptor (hIGF-1R) and has a mitogenic potential like native human insulin but with reduced potency (Moyers et al. 2021).

3.1.3 Pharmacology

Obviously, a long half-life is essential to make insulins suited for once-weekly dosing. A longer half-life will reduce peak-trough fluctuations, but will also be associated with more time to reach steady state after insulin initiation or dose changes. In general, it takes more than 3 half-lives to reach clinical steady state defined as trough concentrations exceeding 90% of the final plateau level (Heise et al. 2016).

For insulin icodec, a half-life of 196 h (~8.2 days) was presented (Hövelmann et al. 2020), whereas the reported half-life of BIF of 17 days was more than twice as long (Heise et al. 2021). In fact, the half-life of BIF might be even longer considering its peak-to-trough ratio of 1.14 over 1 week (Heise et al. 2021). As there is a direct correlation between half-life, peak-to-trough interval and peak-to-trough ratio ($\text{half} - \text{life} = \ln(2) \bullet \frac{\text{peak}-\text{to}-\text{trough interval}}{\ln(\text{peak}-\text{to}-\text{trough ratio})}$), it is easy to calculate that the half-life of BIF is in the range of 3 weeks. The half-life of insulin icodec would then translate into a peak-to-trough ratio of 1.81 in a one-week treatment interval, which is very comparable to the peak-to-trough ratio of insulin glargine U100 over 24 h (Fig. 2) (Becker et al. 2015).

With these reported pharmacological characteristics, both insulins should be suited for once-weekly dosing. BIF with its longer half-life will have very low changes in its glucose-lowering effect over 1 week, but will certainly require a loading dose to reach steady state in a reasonable time frame (without loading dose, it will take more than 3 months to reach "clinical" steady state). In contrast, insulin icodec will reach steady state much faster (in a bit more than 3 weeks without a loading dose), but will have higher fluctuations in its metabolic action over 1 week (Fig. 2, lower panel) (Heise 2021a). While this might be less important for patients with type 2 diabetes, the variability in basal insulin effect might be more difficult to cope with for patients with type 1 diabetes who might have to use lower bolus insulin doses in the first days post-dosing and higher doses towards the end of the dosing interval.

3.1.4 Clinical Data

Several phase 2 clinical studies demonstrated the potential of insulin icodec in patients with type 2 diabetes (data in people with type 1 diabetes have not yet been presented). The first study with a randomised double-blind design was done in insulin-naïve patients who received either once-weekly subcutaneous icodec or once-daily subcutaneous glargine U100 over 26 weeks (Rosenstock et al. 2020). The starting dose of insulin icodec was 70U once weekly, comparable to the 10U once-daily starting dose of insulin glargine. All insulin doses were adjusted once a week with the aim to achieve a pre-breakfast self-measured plasma glucose of 70–108 mg/dl (3.9–6.0 mmol/l). Starting from baseline HbA1c-levels of about 8% mean end of treatment (EOT) levels were 6.69% with icodec and of 6.87% with glargine with a trend of statistical superiority for insulin icodec ($p = 0.08$). In line with these HbA1c-results the mean self-measured plasma glucose levels were lower with icodec than with glargine (difference −7.9 mg/dl, 95% confidence interval −14.10 to −1.62 mg/dl) as was the time with glucose level in range of 70–140 mg/dl in the last 2 weeks of treatment (66.1% vs. 60.7%). While the incidence and rates of confirmed level 1 hypoglycaemia (blood glucose level <70 mg/dl or <3.9 mmol/l) were significantly higher with icodec than with glargine, the incidence and rates of the clinically more relevant level 2 hypoglycaemia (blood glucose level <54 mg/dl or <3.0 mmol/l) were similar. Weekly insulin doses were 19% lower with icodec during the last 2 weeks of treatment than with glargine, however, this finding could

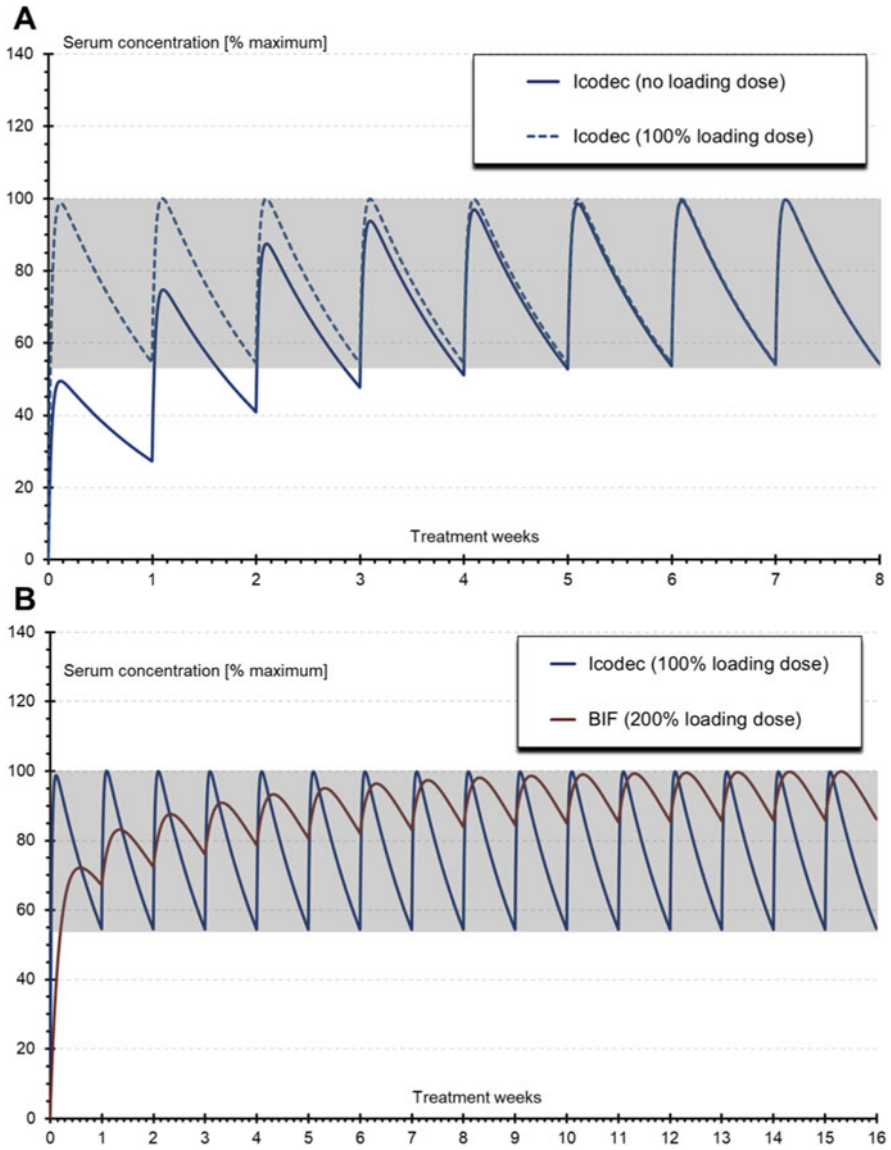

Fig. 2 PK-simulations of insulin icodec with and without loading dose and BIF (with loading dose). Simulated pharmacokinetic profiles of insulin icodec when started with and without a loading dose at first administration (panel **a**) and of insulin icodec and basal insulin Fc BIF with a loading dose (panel **b**). A double dose has been simulated as loading dose for insulin icodec, and a triple dose for BIF. The grey shaded area shows the simulated peak-trough fluctuations at steady state with once-daily injected insulin glargine U100

not be replicated in other studies. Both insulins were well tolerated and no unexpected safety findings occurred. A body weight gain of about 1.5 kg was observed in either treatment group.

These results are comparable to those obtained in patients already treated with basal insulin and at least one oral antidiabetic agent (Bajaj et al. 2020). In this study, a 100% loading dose was used in one icodec arm (icodec+LD, i.e., the first icodec dose was doubled) which resulted in better outcomes compared with the icodec arm with no loading dose (icodec NLD). In particular, time in range measured with continuous glucose monitoring (CGM) during the last 2 weeks of the 16 week treatment period was significantly higher for icodec+LD than that achieved with the comparator daily glargine U100 (72.9% vs. 65.0%, $p = 0.01$) and numerically higher than with icodec NLD (66.0%, $p = 0.75$). Similar to the results in insulin-naïve patients, HbA1c-values trended to be lower with icodec+LD vs. glargine (EOT difference −0.23%, $p = 0.08$), whereas hypoglycaemia incidence and rates as well as insulin doses and adverse event rates were similar across treatment arms. As expected a transient elevation of fasting plasma glucose was observed in the icodec NLD arm. This increase could be avoided with the loading dose.

The question how to best titrate doses of a once-weekly insulin was investigated in a third study which compared several titration algorithms for insulin icodec in insulin-naïve patients with type 2 diabetes (Lingvay et al. 2020). The algorithm with the lowest target fasting plasma glucose (FPG) target (3.9–6.0 mmol/l vs. 4.4–7.2-mmol/l with the other algorithms) resulted in better HbA1c-levels, but also in considerably higher rates of hypoglycaemia. Perhaps the best results were seen with algorithm B where weekly icodec doses were adjusted by ±28U based on the three most recent FPG values. This algorithm led to numerically higher time in range and lower HbA1c with comparable hypoglycaemia rates versus algorithm A where icodec dose changes were limited to 21U.

The only published clinical study with BIF was done in patients with type 2 diabetes treated with oral antidiabetic drugs and basal insulin (Frias et al. 2021b). Patients were randomised to two BIF-groups (with different dosing algorithms targeting relatively high fasting serum glucose (FSG) concentrations of ≤140 mg/dl and ≤120 mg/dl) or to insulin degludec once daily (target FSG ≤100 mg/dl). The treatment duration was 32 weeks. Both BIF-arms achieved non-inferior HbA1c levels to degludec, although mean FSG-concentrations were higher (by study design). Importantly, hypoglycaemic events ≤70 mg/dL (3.9 mmol/L) occurred less often with BIF than with degludec (a relative risk reduction of 25–26%), particularly nocturnal events (where the relative risk reduction was 33–43%). However, level 2 hypoglycaemic rates were not significantly different between treatment arms, although slightly lower in the BIF-arms. The number of adverse events including serious adverse events was comparable across treatments.

While these first clinical study data certainly demonstrate that the use of once-weekly insulins is feasible and safe in people with type 2 diabetes, clinical advantages are limited to non-significant trends towards slightly improved HbA1c-levels (icodec) and reduction in (nocturnal) level 1 hypoglycaemia (BIF). Several

analyses showed that the duration of hypoglycaemic events was not different between once-weekly insulins and once-daily insulins (Heise et al. 2021; Silver et al. 2021), however, clinically significant (level 2) hypoglycaemia was not reduced versus once-daily insulins. Clearly, phase 3 data will have to be awaited before a fair evaluation of the clinical potential of once-weekly insulins will be possible. It is easy to predict, though, that a mere reduction in the number of insulin predictions will not suffice in most countries for coverage of once-weekly insulins by public health insurance (unless costs will be on par with once-daily insulins). Furthermore, it remains to be seen whether or not once-weekly insulins are suitable at least for a subset of patients with type 1 diabetes.

Despite these current limitations, there seems to be a future of once-weekly insulins in combination with once-weekly incretins which would certainly facilitate treatment intensification in people with type 2 diabetes. For both BIF and icodec a combination with dulaglutide and semaglutide, respectively, has been proposed to be feasible. A study with the combination of insulin icodec and semaglutide (IcoSema) has been started (https://clinicaltrials.gov/ct2/show/NCT05013229, access of 15th Oct 2021), but results have not yet been available.

3.2 Oral Insulins

3.2.1 Background

While once-weekly insulins might be preferred over once-daily injections, it clearly is the oral administration route that is the preferred way of drug intake. For insulins, the advantage of oral administration goes even beyond mere convenience and ease of therapy. The oral absorption of insulin, similar to endogenous insulin, will achieve high insulin concentration in the portal vein before reaching peripheral tissues. Thus, orally absorbed insulin will have a hepato-preferential action avoiding over-insulinisation of peripheral tissues and thereby potentially reducing the risk of hypoglycaemia (Heise 2021a). And as semaglutide is already available as oral preparation, an oral combination of incretin and insulin might also be feasible, provided that oral insulin administration works.

Indeed, oral application of insulin was tested very soon after its first subcutaneous administration in humans. The first publication was published in 1923 and was the first of many testimonials of the major challenges of oral insulin administration, in particular high variability in insulin absorption and poor bioavailability (Harrison 1923).

Nevertheless, oral insulin absorption has been shown to be feasible. A glucose clamp study in patients with type 2 diabetes demonstrated a bioavailability of 26% in the first hour post-dosing, whereas bioavailability over the 6 h of the experiment was only 2%. This difference between early and total bioavailability of oral insulin was due to a fast onset of absorption with high peak serum insulin levels and a considerably shorter duration of action compared with s.c. regular human insulin (Kapitza et al. 2010). This pharmacokinetic (PK)/pharmacodynamics (PD) profile with a fast onset and short duration of action would predestine oral insulin for prandial insulin

coverage, perhaps even allowing postprandial intake. Unfortunately, however, oral insulin preparations have a pronounced "food effect", so that absorption is considerably lower when given with or shortly after a meal (Khedkar et al. 2020; Halberg et al. 2019a). Therefore, a dosing-meal interval has to be used with prandial oral insulin formulations, and it has been shown that insulin absorption improves with increasing duration of such an interval. However, a longer administration-meal interval also bears a risk of early hypoglycaemia (due to a strong early insulin effect before oral glucose absorption) and of late postprandial hyperglycaemia (because of declining insulin absorption, but on-going glucose absorption) (Heise 2021a; Khedkar et al. 2020).

3.2.2 Prandial Oral Insulins

Prandial oral insulin formulations have been described in excellent previous reviews (Zijlstra et al. 2014). One such formulation is IN-105 or insulin tregopil, an insulin analogue with a polyethylene glycol side chain at position B29 and sodium caprate as absorption enhancer (Khedkar et al. 2010). Due to its rapid absorption, short duration of action and its food effect (Khedkar et al. 2019), insulin tregopil should be administered 10–30 min before meal intake (Khedkar et al. 2010, 2020). However, this did not suffice to achieving a placebo-adjusted HbA1c reduction of 0.7%, the predefined primary efficacy endpoint in an early phase 3 clinical trial in patients with type 2 diabetes. Insulin tregopil did induce significant improvements in early postprandial glucose levels, though (Zijlstra et al. 2014). In another 24-week treatment study in 91 people with type 2 diabetes comparing 30 and 45 mg of insulin tregopil (to be administered 10 min before meal intake) with s.c. insulin aspart (injected within 5 min before meal intake) insulin tregopil even showed numerical deteriorations in HbA1c (increase by 0.11–0.15%), whereas insulin aspart improved HbA1c by 0.78% (https://clinicaltrials.gov/ct2/show/results/NCT03430856, access 11 Oct 2021). Hypoglycaemia incidence was comparable across treatment groups (83–87% of patients). A study in people with type 1 diabetes is on-going (https://clinicaltrials.gov/ct2/show/NCT04141423, access 11 Oct 2021).

Another oral insulin in development is ORMD-0801, formulated in an enteric-coated capsule with further unspecified adjuvants that shall both protect the insulin from degradation in the gastrointestinal tract and enhance its absorption (Zijlstra et al. 2014). While the number of clinical trials with ORMD-0801 registered on https://clinicaltrials.gov is quite impressive (to date 7 completed, 5 recruiting and 3 not yet recruiting), only one of the more recently completed studies has been published (Eldor et al. 2021). Like most other published studies with ORMD-0801, this study only compared to placebo rather than to a s.c. insulin, which makes it quite difficult to evaluate clinical efficacy. Nevertheless, the results of this publication (the "largest Phase II, placebo-controlled study conducted with ORMD-0801 to date" in 188 patients with type 2 diabetes) raise some doubts that ORMD-0801 will become a viable treatment option for people with diabetes. The pooled results of patients randomised to 16 mg or 24 mg ORMD-0801 (administered at bedtime, at least 2 h after the last evening meal and at least 6 h before the next meal) in addition to pre-exiting metformin therapy did not show any meaningful improvements in

HbA1c or baseline weighted mean night-time CGM readings, even though the observed small increase in the latter parameter was significantly lower than the increase observed with placebo (Eldor et al. 2021). The absence of nocturnal hypoglycaemic events in the study might rather be a sign of insufficient insulin absorption than a safety feature. Poor absorption was also indicated by a glucose clamp study comparing two 8 mg ORMD-0801 capsules with three 8 mg capsules and one 16 mg capsule under euglycaemic clamp conditions with tritiated glucose to assess hepatic glucose production (HGP) in 11 people with type 1 diabetes (https://clinicaltrials.gov/ct2/show/results/NCT02535715, access 11 Oct 2021). None of the ORMD-0801 formulations showed increases from baseline plasma insulin concentrations over a period of 240 min, and suppression of HGP was modest in all three treatment arms. In addition, other clinical studies have also only shown modest effects on glycaemic control. In small studies using CGM over 1 or 2 weeks in people with type 1 or type 2 diabetes, ORMD-0801 slightly reduced daytime and night-time glucose levels (by 1.0–1.5 mmol/l or less) and basal insulin doses (in type 1 diabetes) versus placebo (https://clinicaltrials.gov/ct2/show/results/NCT01889667 and https://clinicaltrials.gov/ct2/show/results/NCT02094534, access 11 Oct 2021). Despite these shortcomings FDA approved several phase 3 studies in people with type 2 diabetes which are currently on-going.

Overall, these results show that the challenges of developing prandial oral insulin have not changed since 1923. Variability preventing a clear dose-response relationship in combination with low bioavailability, in particular when given in close temporal proximity with food, limits the clinical efficacy of prandial oral insulin (Heise 2021a).

3.2.3 Basal Oral Insulins

A basal oral insulin would solve all issues about the food effect of oral insulin absorption as a basal insulin could be taken far before or after a meal. However, achieving a basal, constant insulin supply from an orally administered insulin is a major challenge. This challenge was addressed with the long-acting basal insulin analogue 338 (I338) formulated with a gastrointestinal permeation enhancer (GIPET I) and an absorption enhancer (sodium caprate) (Halberg et al. 2019b; Kjeldsen et al. 2021). I338 contains an 18-carbon fatty diacid which is attached via a linker to the insulin molecule and allows reversible binding to albumin. Thus, I338 is absorbed quickly but released only slowly from the albumin pool resulting in a half-life of up to 70 h at steady state (Kjeldsen et al. 2021). This allows meal-independent dosing of I338 which, however, also shows a food effect I338 (Halberg et al. 2019a). A double-blind, double-dummy phase 2 clinical study compared oral I338 with insulin glargine U100 s.c. once daily for 8 weeks in insulin-naïve patients with type 2 diabetes on metformin therapy with or without other oral antidiabetic agents. I338 was given in a fasted state and patients were asked to refrain from fluid and food intake for 1 h. At the end of treatment, fasting plasma glucose (FPG) levels (the primary endpoint) and 10-point plasma glucose profiles were not significantly different between treatment arms (Fig. 3), neither were HbA1c-levels (although they were numerically lower (with a difference of 0.3%) s.c. insulin glargine.

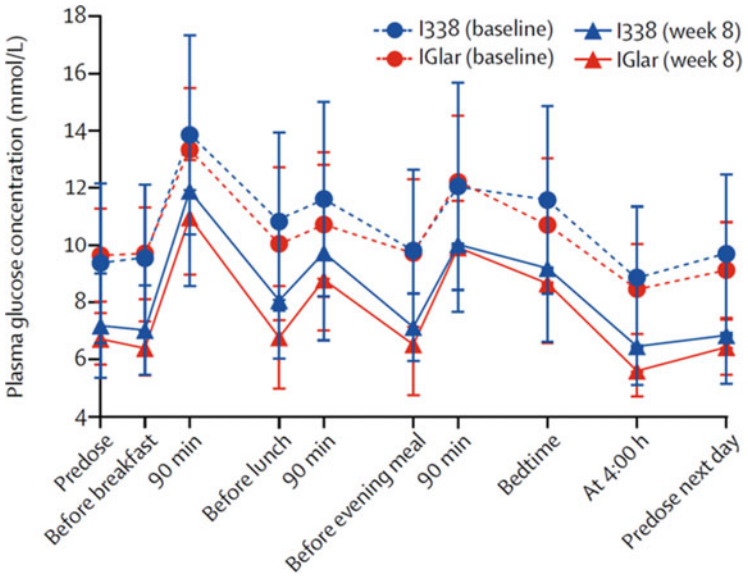

Fig. 3 Glucose control with oral insulin. 10-point plasma glucose concentration profiles at baseline and after 8 weeks of treatment with oral insulin I338 or subcutaneous insulin glargine U100 (IGlar). Data are arithmetic mean (SD). Reprinted from Halberg et al. (2019b) with permission

Interestingly, within-patient variability in FPG concentration was higher by 52% in the I338 group than in the insulin glargine group, but again this difference did not reach statistical significance and was already present at baseline.

Overall, this study was the first demonstrating that oral insulin therapy might be feasible, at least in people with type 2 diabetes when using a basal oral insulin with a long half-life. While food effect and high variability of oral insulins could be successfully overcome with this approach, the issue of a low bioavailability remained (I338 doses were approximately 58 times higher than insulin glargine doses at the end of treatment). While this bioavailability was still higher than that of oral semaglutide (Buckley et al. 2018), it led to discontinuation of the development of I338 as "production of the required quantities of I338 for wide public use was deemed not commercially viable" (Halberg et al. 2019b).

Therefore, despite having shown proof of concept, the further development of oral basal insulin seems unclear. Approaches to increase bioavailability have not yet reached clinical stage (Abramson et al. 2019). Likewise, new strategies, e.g. using oral insulin early in type 2 diabetes to improve beta-cell dysfunction and insulin sensitivity or even achieve remission (Kramer et al. 2013) have not been investigated to date.

4 Summary and Conclusions

It is easy to predict that future diabetes therapy will comprise of tirzepatide and once-weekly insulins in the nearer future, whereas it will take much more time before other co-agonists or triple agonists or other insulin developments (like hepato-preferential, oral or glucose-responsive insulins) will be part of a clinical routine. Many other developments have not been addressed in this paper because clinical data are still outstanding. Although pharmacological diabetes therapy was introduced with the discovery of insulin 100 years ago (Banting et al. 1922), the development of antidiabetic drugs is still on-going. Many promising data of novel antidiabetic drugs clearly indicate that therapy of people with diabetes will become easier, safer and more efficacious in the next years.

Conflict of Interest TH is shareholder of the private research institute Profil which received research funds from Adocia, Afon Technology, Astra Zeneca, Biocon, Boehringer Ingelheim, Eli Lilly, Gan Lee Pharmaceuticals, Johnson & Johnson, Julphar, Mylan, Nestlé, Neuraly, Nordic Bioscience, Novo Nordisk, Sanofi and Zealand Pharma. TH received speaker honoraria and travel grants from Eli Lilly and Novo Nordisk, and was a paid member of advisory panels for Novo Nordisk and Valbiotis.

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Novel Approaches to Restore Pancreatic Beta-Cell Mass and Function

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Abstract

Beta-cell dysfunction and beta-cell death are critical events in the development of type 2 diabetes mellitus (T2DM). Therefore, the goals of modern T2DM management have shifted from merely restoring normoglycemia to maintaining or regenerating beta-cell mass and function. In this review we summarize current and novel approaches to achieve these goals, ranging from lifestyle interventions to N-methyl-D-aspartate receptor (NMDAR) antagonism, and discuss the mechanisms underlying their effects on beta-cell physiology and glycemic control. Notably, timely intervention seems critical, but not always strictly required, to maximize the effect of any approach on beta-cell recovery and disease progression. Conventional antidiabetic medications are not disease-modifying in the sense that the disease does not progress or reoccur while on treatment or thereafter. More invasive approaches, such as bariatric surgery, are highly effective in restoring normoglycemia, but are reserved for a rather small proportion of obese individuals and sometimes associated with serious adverse events. Finally, we recapitulate the broad range of effects mediated by peripheral NMDARs and discuss recent evidence on the potential of NMDAR antagonists to be developed as a novel class of antidiabetic drugs. In the future, a more refined assessment of disease risk or disease subtype might enable more targeted therapies to prevent or treat diabetes.

Keywords

Beta-cell recovery · Diabetes mellitus · Glycemic control · NMDA receptor antagonists · Pancreatic islets

1 Introduction

In the developed pancreas, beta-cells are capable to adapt to changes in secretory demands. For example, in individuals with obesity, the pancreas initially responds by expanding the mass of beta-cells to approximately 20–50% more than that of normal weight individuals and by markedly increasing the rate of insulin secretion (Weir et al. 2020). The compensatory adaption of beta-cell mass is the result of self-replication of existing beta-cells, beta-cell enlargement, neogenesis of beta-cells from precursor cells, and possibly transdifferentiation of other cells (such as alpha-cells) into beta cells (Bonner-Weir et al. 2010; Weir et al. 2020). Type 2 diabetes mellitus (T2DM) develops, when beta-cells fail to secrete enough insulin for the

current degree of insulin sensitivity. This is either due to a combination of insulin secretory defects and insufficient beta-cell mass expansion and/or increased beta-cell death and dedifferentiation upon metabolic insults (Weir et al. 2020). Notably, compared to nondiabetic individuals, lean and obese individuals with prediabetes or T2DM have been found to have a deficit in relative beta-cell volume, an estimate of beta-cell mass, of 40–60% (Butler et al. 2003).

To date, there are no means to restore beta-cell mass in human individuals with diabetes other than pancreas or islet transplantation, albeit drug-dependent promotion of human beta-cell proliferation has been demonstrated in preclinical models (Dai et al. 2017; Dirice et al. 2016; Shen et al. 2015; Wang et al. 2015). Therefore, there is great interest in finding less invasive interventions that either preserve beta-cell mass in at-risk individuals or regenerate beta-cell mass in those with recent-onset or long-standing disease.

2 Lifestyle Intervention

Comprehensive lifestyle modification is a fundamental aspect of diabetes care and should be emphasized along with any pharmacologic therapy for the treatment of T2DM (ADA 2019a). Lifestyle intervention that aims at reducing body weight is highly effective in preventing diabetes in at-risk individuals and on returning those with prediabetes or T2DM to normal glucose regulation (DPP Research Group 2015; Knowler et al. 2002; Lean et al. 2019; Lindström et al. 2013; Perreault et al. 2009, 2012; Tuomilehto et al. 2001).

2.1 Lifestyle Intervention and Diabetes Remission

Several trials demonstrated that T2DM can be reversed upon substantial weight loss and that avoidance of subsequent weight regain associates with sustained remission (Lean et al. 2019; Lim et al. 2011; Steven et al. 2016a). Most recently, the Diabetes Remission Clinical Trial DiRECT, which is currently conducted at primary care practices in the United Kingdom (UK), demonstrated that 1 and 2 years after initiation of a structured weight-management program 46% and 36% of individuals with T2DM of less than 6 years duration achieved remission (defined as HbA1c less than 6.5% after at least 2 months off all antidiabetic medications), compared to 4% and 3% of control participants, respectively (Lean et al. 2018, 2019). Notably, among those individuals in remission, approximately two thirds achieved normal fasting plasma glucose and HbA1c level (Taylor et al. 2019).

2.2 Mechanisms Underlying the Effects of Lifestyle Intervention on Glycemic Control

In obese individuals with T2DM, only those weight loss intervention studies that result in a weight reduction of >5% elicit beneficial effects on HbA1c, lipids, and blood pressure (Franz et al. 2015). The effect of weight loss on glycemic control appears to be largely driven by a reduction of ectopic fat in the liver and in the pancreas and, critically, the ability to recover first phase insulin release (Taylor et al. 2018). Importantly, in T2DM, the accumulation of fat in the liver and secondarily in the pancreas is thought to maintain a vicious cycle promoting the development of insulin resistance, dysglycemia, and ultimately T2DM (the twin cycle hypothesis). In individuals with T2DM, liver fat content and hepatic insulin resistance have been shown to normalize within days after initiation of a low-caloric diet, while pancreatic fat content declined more gradually, probably resulting in a recovery of first phase insulin release by 8 weeks (Lim et al. 2011; Steven et al. 2016b).

More recently, DiRECT confirmed profound changes in lipid metabolism along with substantial reductions in body weight achieved 1 year after initiation of the weight-management program. Large reductions in hepatic and pancreatic fat content and a fall in total plasma triglyceride level were observed among all participants, irrespective of their response in terms of glycemic control (Taylor et al. 2018).

Besides the large beneficial effects of intensive lifestyle interventions (ILS) on diabetes prevention and diabetes remission (DPP Research Group 2015; Knowler et al. 2002; Lean et al. 2019; Lindström et al. 2013; Perreault et al. 2009, 2012; Tuomilehto et al. 2001), up to 50% of individuals do not respond to lifestyle interventions in terms of glucose control, despite adequate weight loss. This is equally true for individuals with prediabetes and those with type 2 diabetes (Lean et al. 2018; Schmid et al. 2017). In DiRECT, similar baseline characteristics were observed among those individuals that achieved diabetes remission (responder) and those who did not (non-responder), apart from lower fasting plasma insulin level, lower plasma alanine aminotransferase level, higher HbA1c, and modestly longer diabetes duration in non-responders, suggesting a more advanced stage of disease. Notably, additional metabolic studies revealed similar reductions in body weight, liver and pancreatic fat content and triglyceride plasma concentrations among responders and non-responders. However, only responders were capable to recover first phase insulin release and to maintain increased first phase insulin secretion during the weight maintenance phase, while no change from baseline in beta-cell function was observed in non-responders. It has therefore been concluded that the ability to respond to a lifestyle intervention in terms of glucose control is intrinsic to the beta-cell, and clinical data suggest a more advanced, irreversible stage of beta-cell dysfunction in non-responders, even among a group of individuals with rather short diabetes duration as in DiRECT (<6 years) (Steven et al. 2016a; Taylor et al. 2018).

3 Pharmacological Approaches

Several classes of antidiabetic drugs with different mechanisms of action are available for use in individuals with T2DM. The biguanide metformin is the preferred initial pharmacologic agent for the treatment of T2DM and the most commonly prescribed drug for this condition worldwide (ADA 2019b; Flory and Lipska 2019). Current recommendations on the use of additional oral or injectable antihyperglycemic agents account for the prevailing cardiovascular risk profile, renal function, potential side effects, and treatment costs (ADA 2019b). Pharmacological approaches to maintain or enhance beta-cell function are manifold and include reducing the secretory demand on beta-cells by improving insulin sensitivity (metformin, thiazolidinediones), reversal of glucotoxicity through insulin-independent mechanisms (SGLT2 inhibitors), induction of beta-cell rest (exogenous insulin), and promotion of beta-cell proliferation and survival (GLP-1 receptor agonists, twincretins).

3.1 Insulin

Trials of insulin therapy in adults with impaired glucose tolerance or early T2DM support the concept that insulin has favorable effects on beta-cell function, disease progression, and glycemic remission (Gerstein et al. 2012; Li et al. 2004; Weng et al. 2008). In individuals with newly diagnosed T2DM, short-term intensive glycemic control with insulin therapy (IT) has been shown to increase the likelihood of diabetes remission, defined by a fasting plasma glucose (FPG) of less than 126 mg/dl and a postprandial glucose (PPG) of less than 180 mg/dl. While almost half of the individuals treated with insulin were in clinical remission at 1 year, this was only true for approximately one fourth of individuals treated with sulfonylurea and/or metformin (Li et al. 2004; Weng et al. 2008). IT was furthermore associated with improved beta-cell function at 1 year, while intensive glycemic control with sulfonylurea and/or metformin was not (Weng et al. 2008). However, insulin therapy fails to sufficiently restore beta-cell function to induce long-lasting clinical remission, as evidenced by the declining remission rates following IT that were 73%, 67%, 47%, and 42% at the 3rd, 6th, 12th, and 24th month (Fig. 1), respectively (Li et al. 2004).

3.2 Metformin, Sulfonylurea, Thiazolidinediones

Metformin is a non-selective drug that promotes the uptake of glucose by peripheral tissues, particularly the skeletal muscle, and primarily inhibits hepatic gluconeogenesis, thus improving insulin sensitivity and decreasing hepatic glucose output. It has a good safety profile, with a low risk of hypoglycemia, and the potential for some weight loss (ADA 2019b; Sciannimanico et al. 2020).

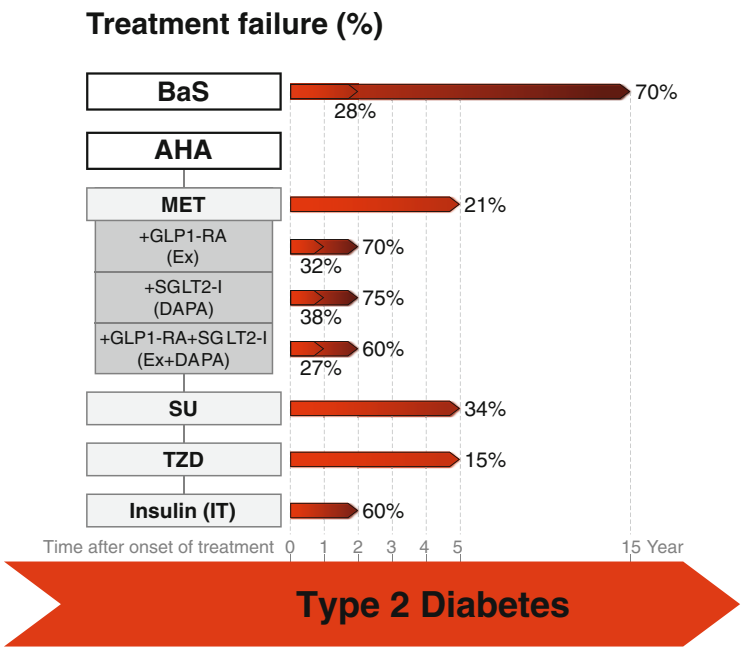

Fig. 1 Treatment failure in individuals with type 2 diabetes mellitus. Treatment failure following either bariatric surgery (BaS) or treatment with antihyperglycemic agents (AHA) in individuals with type 2 diabetes (T2DM). Please note that direct comparison is hampered by different study designs, particularly baseline characteristics and definitions of treatment failure. Baseline characteristics and definitions are as follows. BaS: obese individuals with T2DM that underwent bariatric surgery (baseline characteristics: diabetes duration 2.9 years, BMI 42.1 kg/m^2, age 48 years); diabetes remission defined as FPG < 110 mg/dl and no diabetes medication (Sjöström et al. 2014). MET, SU, and TZD: dug-naïve obese individuals with T2DM randomly assigned to receive either metformin (MET), the sulfonylurea glyburide (SU) or the thiazolidinedione rosiglitazone (TZD) (baseline characteristics: diabetes duration mostly [>96%] ≤2 years, BMI 32.2 kg/m^2, age 57 years, HbA1c 7.4%); treatment failure defined as confirmed level of FPG of more than 180 mg/dl (Kahn et al. 2006). Insulin: drug-naïve individuals with "newly diagnosed" T2DM who underwent short-term intensive glycemic control with insulin therapy (IT) (baseline characteristics: BMI 25 kg/m^2, age 48.9 years, HbA1c 10.1%); glycemic relapse defined as confirmed FPG >126 mg/dl or 2-h blood glucose >180 mg/dl, glycemic remission defined as "percentages of the subjects maintaining near euglycemia on diet alone" (Li et al. 2004). GLP-1RA, SGLT2-I: results from DURATION-8, obese individuals with T2DM inadequately controlled with metformin alone randomly assigned to receive the GLP-1 receptor agonist (GLP-1RA) exenatide (Ex), or the SGLT2 inhibitor (SGLT2-I) dapagliflozin (DAPA), or both (Ex+DAPA) (baseline characteristics: diabetes duration 7.4 years, BMI 32.7 kg/m^2, age 54.2 years, HbA1c 9.3%); diabetes remission defined as proportion of individuals who achieved an HbA1c <7.0% (Jabbour et al. 2018b, 2020)

In contrast, sulfonylureas are associated with weight gain and risk of hypoglycemia, which however is less frequent than with insulin therapy. The incidence of severe hypoglycemia is furthermore lower with the newer-generation sulfonylureas (glipizide, glimepiride, and gliclazide) compared to glyburide (Khunti et al. 2018).

Sulfonylureas are currently prescribed as second- or third-line therapy for the management of T2DM (Davies et al. 2018).

In 1999 the thiazolidinediones (TZDs) rosiglitazone and pioglitazone were approved by the FDA for the treatment of T2DM (Quianzon and Cheikh 2012). In 2010, however, rosiglitazone was withdrawn from the market because of its potential to increase the risk of myocardial infarction and death from cardiovascular diseases (Nissen and Wolski 2007). TZDs primarily improve insulin sensitivity. They activate the peroxisome proliferator activator receptors (PPAR) and thus facilitate peripheral glucose uptake in numerous tissues (Chaudhury et al. 2017). TZDs have a high glucose-lowering efficacy, however, they are also associated with fluid retention, heart failure, weight gain, bone fracture and, possibly, bladder cancer (Davies et al. 2018).

3.2.1 Effects on Glycemic Control and Beta-Cell Function in Individuals with T2DM

Head-to-head trials comparing the biguanide metformin, the sulfonylurea glyburide and the TZD rosiglitazone in recently diagnosed drug-naïve individuals with T2DM revealed that the durability of glycemic control and the overall preservation of beta-cell function is greatest with rosiglitazone and least with glyburide, while metformin is intermediate (Kahn et al. 2006, 2011). The maximal treatment effect on HbA1c was achieved at 12 months in individuals treated with either metformin or rosiglitazone, and at 5 months for those treated with glyburide. Thereafter, HbA1c progressively increased in all treatment groups at a rate ranging from 0.07% per year to 0.24% per year, the rate of increase being greatest with glyburide and least with rosiglitazone. Notably, at the 4-year evaluation, less than half of all individuals in each treatment group achieved an HbA1c level below 7%, and at 5 years, monotherapy failure, defined as a confirmed fasting plasma glucose of more than 180 mg/dl, was 15% with rosiglitazone, 21% with metformin, and 34% with glyburide (Fig. 1) (Kahn et al. 2006).

Additional studies in the same cohort revealed that rosiglitazone slows the rate of loss of beta-cell function and improves insulin sensitivity to a greater extent than metformin or glyburide, consistent with its greater effect on glycemic control (Kahn et al. 2006). Importantly, while the effect of rosiglitazone on insulin sensitivity was sufficient to compensate for the progressive decline in insulin response yielding an overall preservation of beta-cell function, as determined by the oral disposition index (oDI), a measure of beta-cell function relative to insulin sensitivity, glyburide treatment led to a rapid and progressive decline in oDI. Although metformin also delayed the progressive decline in beta-cell function, the overall effect on oDI was minimal at 4 years of follow-up (Kahn et al. 2011). In sum, it can be concluded from these trials that neither rosiglitazone nor metformin or glyburide constantly maintain beta-cell function and glycemic control in the majority of individuals with T2DM.

3.3 GLP-1 Receptor Agonists and DPP-4 Inhibitors

3.3.1 The Physiological Effects of GLP-1

Glucagon-like peptide-1 (GLP-1) is an intestinal hormone (incretin) that is released from enteroendocrine cells in the distal small bowel and colon upon nutrient ingestion (Drucker 2018). It stimulates insulin secretion and inhibits glucagon release in a glucose-dependent manner, delays gastric emptying, improves satiety and thus promotes weight loss. GLP-1 exerts its insulinotropic actions through G-protein-coupled receptors that are expressed in pancreatic islets, as well as kidney, lung, heart, and multiple regions of the peripheral and central nervous system (CNS) (Drucker 2018). Within islets, the GLP-1 receptor is predominantly localized to beta-cells, although GLP-1 receptor expression within a subset of human islet alpha-cells and human delta-cells has also been reported (Drucker 2018; Waser et al. 2015). GLP-1 receptor signaling within beta-cells is essential for glucose homeostasis (and for the pharmacological effects of GLP-1 receptor agonists), albeit central GLP-1 receptors also contribute to the effects of GLP-1 on the regulation of glycemia and energy balance, either by directly regulating appetite and food intake or by indirectly regulating peripheral glucose homeostasis, gastric emptying, lipogenesis, and thermogenesis through the activation of the afferent nervous system (Drucker 2018; Lamont et al. 2012).

3.3.2 Effects on Beta-Cell Function and Mass in Preclinical Models

Beyond its glucoregulatory actions, GLP-1 has been shown to regulate beta-cell mass in experimental models of diabetes through the induction of beta-cell proliferation and differentiation (i.e., neogenesis) and the protection against apoptotic injury (Farilla et al. 2002; Kapodistria et al. 2018; Li et al. 2003; Shimoda et al. 2011; Wang and Brubaker 2002; Xu et al. 1999; Yusta et al. 2006). For example, in a partial pancreatectomy rat model of T2DM, the GLP-1 receptor agonist (GLP-1RA) exendin-4 expands beta-cell mass by stimulating beta-cell proliferation and neogenesis, i.e. the differentiation of ductal progenitor cells into beta-cells (Xu et al. 1999). GLP-1 also promotes the differentiation of human pancreatic islet-derived progenitor cells into functioning beta-cells in vitro (Abraham et al. 2002).

Conversely, genetic deletion of the GLP-1 receptor markedly impairs beta-cell recovery in partially pancreatectomized mice, indicating that GLP-1 receptor signaling is required for the adaptive regeneration of beta-cell mass in response to increased metabolic demands (De León et al. 2003). Recently, the GLP-1RA liraglutide has been shown to facilitate pancreatic neogenesis in mice, i.e. the formation of immature pancreatic lobes containing rapidly proliferating endocrine cells, acinar cells, pancreatic ducts, and blood vessels (Deng et al. 2020). It should however be noted that the proliferative capacity of beta-cells has been shown to decrease with age. A variety of stimuli, including the GLP-1RA exendin-4, induce beta-cell proliferation in young mice and in juvenile human islets, but have no effect in aged mice or in adult human islets (Dai et al. 2017; Drucker 2018; Tschen et al. 2009, 2011). In addition, experiments with human pancreatic islets transplanted into

the anterior eye chamber of mice indicate that long-term treatment with GLP-1RA (that is, liraglutide) results in beta-cell dysfunction (Abdulreda et al. 2016).

The molecular mechanisms underlying GLP-1 action on pancreatic islets are manifold and not yet fully elucidated. The effects of GLP-1 on proliferation, differentiation, and cell survival of pancreatic beta-cells are dependent on the expression of the pancreatic duodenal homeobox-1 (PDX-1) transcription factor, which plays a critical role in pancreas development. Beta-cell specific deletion of *Pdx1* increases the rate of apoptosis, and exendin-4 fails to stimulate beta-cell proliferation or inhibit apoptosis in *Pdx1*-deficient islets in vivo (Drucker 2006; Hui et al. 2001; Li et al. 2005). Recently, GLP-1 has been shown to act on alpha-cell transdifferentiation to beta-cells via induction of fibroblast growth factor 21 (FGF21) expression in alpha-cells and a subsequent increase in beta-cell transcription factors, e.g. PDX-1 and neurogenin-3 (Ngn-3) (Lee et al. 2018). Notably, alpha-cells have been shown to produce GLP-1 under conditions of beta-cell expansion, such as during pregnancy or in *ob/ob* and *db/db* mice (Kilimnik et al. 2010). It has therefore been assumed that circulating GLP-1 and GLP-1 locally produced by alpha-cells may contribute to beta-cell compensation under diabetogenic conditions and promote alpha-cell transdifferentiation to new beta-cells via FGF21. Please note that the GLP-1 induced signal transduction pathways involving beta-arrestin-1, beta-catenin, and IGF-1 receptor signaling have been reviewed elsewhere (Campbell and Drucker 2013).

3.3.3 Effects on Glycemic Control and Beta-Cell Function in Individuals with T2DM

The effects of GLP-1 on glycemic control, beta-cell proliferation, neogenesis, and survival triggered the development of incretin-based therapies for the treatment of T2DM. Native GLP-1 is proteolytically degraded and inactivated by the ubiquitous protease dipeptidyl peptidase-4 (DPP4) and thus rapidly eliminated from the circulation within a half-life of approximately 2 min. Therefore GLP-1RA, resistant to proteolytic inactivation and with slower elimination kinetics, have been developed (Gupta 2013; Nauck et al. 2020). Exenatide was the first GLP-1RA approved for the treatment of T2DM by the FDA in April 2005 and by the EMA in November 2006 (Gupta 2013). Meanwhile several GLP-1RA with prolonged half-life have been granted marketing authorizations, including an extended release formulation of exenatide that can be administered once weekly and an oral formulation of semaglutide (Nauck et al. 2020). Yet another approach for enhancing the action of GLP-1 is inhibiting the action of DPP-4, the key enzyme responsible for cleaving and inactivating GLP-1 (DPP-4 inhibitors). GLP-1RA provide superior glycemic control and weight loss compared to DPP-4 inhibitors, given that they are applied at *supraphysiologic* concentrations (Aroda et al. 2012).

An early meta-analysis conducted on the efficacy and safety of incretin-based therapies in adults with T2DM revealed HbA1c reductions of −0.97% and −0.74% for GLP-1RA and DPP4 inhibitors compared to placebo, respectively, following 12–52 weeks of treatment. Furthermore, GLP-1RAs resulted in weight loss (−1.4 kg and − 4.8 kg compared to placebo and insulin, respectively), while DPP4 inhibitors were weight neutral (Amori et al. 2007). Longer-acting GLP-1RA (e.g., liraglutide)

have been shown to be more efficient in terms of glycemic control and weight loss compared to short-acting GLP-1RA (Huthmacher et al. 2020). When added to oral glucose-lowering medications, GLP-1RAs have a slightly better effect on reducing HbA1c than insulin therapy, while significantly reducing hypoglycemic episodes (34% lower with GLP-1RA compared to insulin) and inducing weight loss (Abd El Aziz et al. 2017; Singh et al. 2017). The American Diabetes Association (ADA) and the European Association for the Study of Diabetes (EASD) therefore recommended to preferentially use GLP-1RA as first injectable medication in individuals with T2DM failing on oral agents alone (Davies et al. 2018; Nauck et al. 2020). Importantly, two recent meta-analyses on cardiovascular outcomes comparing GLP-1RA with placebo in individuals with T2DM with and without established cardiovascular disease (CVD) revealed significant reductions in cardiovascular (CV) events, i.e. reductions by 8–16% in the incidence of major adverse cardiovascular events (MACE), stroke, CV- and all-cause mortality and hospitalization for heart failure (Kristensen et al. 2019; Marsico et al. 2020). GLP-1RA furthermore reduced the composite kidney outcome (i.e., new-onset macroalbuminuria, decline in eGFR or increase in serum creatinine, progression to end-stage renal disease, and death attributable to kidney causes) compared to placebo by 17% (Kristensen et al. 2019). Initial concerns that GLP-1RA might increase the risk of pancreatitis and particularly pancreatic cancer as well as thyroid cancer have not been confirmed by large CV outcome trials that did not find an increased risk of severe hypoglycemia, pancreatitis, pancreatic cancer, or thyroid cancer (Butler et al. 2013; Kristensen et al. 2019; Marsico et al. 2020).

In 2002, Zander et al. were the first to demonstrate a beneficial effect of a 6-week course of continuous subcutaneous GLP-1 infusion on beta-cell function as well as insulin sensitivity in individuals with T2DM (Zander et al. 2002). The ability of GLP-1RA to enhance beta-cell function in individuals with T2DM was confirmed in several short- and long-term clinical trials, using both, static and dynamic tests (Bunck et al. 2009, 2011; Chang et al. 2003; Degn et al. 2004; Mari et al. 2006; Retnakaran et al. 2014; van Raalte et al. 2016; Vilsbøll et al. 2008). For example, although in metformin-treated individuals with T2DM, the addition of either insulin glargine or the GLP-1RA exenatide resulted in comparable glycemic control (HbA1c of 6.6% and 6.9% in insulin- and exenatide-treated individuals, respectively), only exenatide improved parameters of beta-cell function, assessed during a standardized mixed meal tolerance test (MMTT) at the end of the 3-year treatment period. Importantly, the improvement in beta-cell function induced by exenatide was statistically independent of changes in body weight (van Raalte et al. 2016). In 2011, Bunck et al. reported modest, albeit significantly sustained improvements in beta-cell function and insulin sensitivity compared to pretreatment values in metformin-treated individuals with type 2 diabetes following 3 years of exenatide and a 4-week off drug period (Bunck et al. 2011). They observed a small improvement in the disposition index, assessed during clamp studies at baseline and 4 weeks after discontinuation of treatment. The improvement in beta-cell function, however, was not associated with better glycemic control, and after 12 weeks off-drug, both, FPG and HbA1c increased to pretreatment values. It has been argued that the substantial

reductions in body weight induced by exenatide (mean weight reduction compared to baseline −5.7 kg) may at least partially account for the improvement in beta-cell function (Drucker 2018). Notably, trials of shorter duration with the GLP1RA liraglutide or exenatide in individuals with T2DM did not reveal any sustained effect on beta-cell function 2–4 weeks after cessation of therapy (Bunck et al. 2009; Retnakaran et al. 2014). Furthermore, in drug-naïve adults with prediabetes or recently diagnosed T2DM, 12 months of liraglutide treatment combined with metformin did not produce sustained benefits in beta-cell function 3 months after treatment withdrawal despite on-treatment benefits (RISE Consortium 2019).

In view of the effects of GLP-1RA on beta-cell proliferation, neogenesis, and survival in preclinical models, the results from clinical trials in individuals with diabetes have been somewhat disappointing in that they argue against an effect of GLP-1RA on beta-cell recovery in humans. This discrepancy has been explained by the loss of basal and GLP-1-stimulated proliferative capacity of aged human beta-cells (Drucker 2018). For example, in islets from juvenile human donors (aged 0.2–9 years), exendin-4 induces expression of calcineurin/NFAT signaling components as well as target genes for proliferation-promoting factors, while the expression of these factors is not affected in islets from adult human donors (aged 20–60 years) exposed to exendin-4 (Dai et al. 2017). Since the expression of intrinsic beta-cell growth regulators, such as the cell cycle inhibitor CDKN2A, was not detectably affected by exendin-4 exposure, a cell-autonomous basis for age-dependent responses of human islets to exendin-4 has been suggested (Dai et al. 2017). These findings imply that human patients of advanced age have little regenerative capacity to increase beta-cell mass and that young rodents do not reliably reflect the regenerative capacity of mature adult human beta-cells (Drucker 2018).

3.4 Twincretins

In recent years, dual unimolecular peptide-based agonists simultaneously targeting the GLP-1- and GIP-receptors, as well as triple agonists additionally targeting the glucagon-receptor have gained much attention as potential novel antidiabetic agents (Usui et al. 2019). Although preclinical studies on the role of GIP-signaling in the regulation of body weight have yielded conflicting results, and although the insulinotropic effect of GIP appears to be blunted in individuals with T2DM, the co-administration of GLP-1 and GIP has been shown to exert additive metabolic effects (Elahi et al. 1994; Finan et al. 2013; Nauck et al. 1993). For example, in healthy human individuals, the insulinotropic effect of GLP-1 and GIP co-administration was significantly higher than that with either hormone alone (Elahi et al. 1994; Nauck et al. 1993). Furthermore, in rodents, the administration of GIP enhanced the weight-lowering efficacy of GLP-1 (Finan et al. 2013). These observations encouraged the development of single-molecule peptides with balanced GLP-1 and GIP receptor agonism (twincretins). In animal models of obesity and diabetes, twincretins have been shown to provide greater metabolic efficacy than

selective GLP-1 receptor agonism, particularly for weight reduction (Finan et al. 2013). Precisely, greater reductions in food intake, body weight, fat mass, and glycemic excursions have been observed in rodents treated with twincretins compared to GLP-1RA alone. Twincretins also preserved beta-cell mass and improved beta-cell functional capacity, as determined by the homeostatic model assessment of fasted insulin to glucose, in rodent models of diabetes compared to control-treated animals (Finan et al. 2013). The beneficial effects of twincretins on body weight and glycemic control have been confirmed in phase 2 clinical trials, and several phase 3 clinical trials are currently conducted (Frias et al. 2017, 2018, 2020; Min and Bain 2021). In individuals with T2DM inadequately controlled with lifestyle intervention or metformin, 23 weeks of treatment with the dual GLP-1/GIP agonist LY3298176 (tirzepatide) led to significantly greater reductions in body weight and HbA1c compared to the GLP-1RA dulaglutide alone and significantly improved beta-cell function and insulin resistance as determined by the homeostasis model assessment (HOMA)2-B and HOMA2-IR (Frias et al. 2018). Recently, confirmatory results have been announced based on the first phase 3 clinical trial with tirzepatide (SURPASS-1). In drug-naïve individuals with T2DM, 40 weeks of tirzepatide dose-dependently reduced HbA1c and body weight. Precisely, among individuals taking the highest dose of tirzepatide, HbA1c and body weight decreased by −2.07% and −9.5 kg (11%) from baseline, respectively, and 50% of participants achieved normal HbA1c level (<5.7%), while no events of severe hypoglycemia were observed. Although these results are encouraging, superior efficacy of dual GLP-1/GIP receptor agonists compared to best-in-class GLP-1RA, cardiovascular safety, and the durability of the twincretin effect on body weight reductions and glycemic control have yet to be proven. Notably, unimolecular triple agonists, simultaneously activating the GLP-1-, GIP-, and glucagon-receptor have also been developed, aiming to combine the twincretin effect with the beneficial effects of glucagon on energy expenditure and lipid metabolism (Hasib 2020). In rodent models of obesity and diabetes, greater metabolic efficacy has been demonstrated with this triagonist compared to the dual GLP-1/GIP receptor agonist (Finan et al. 2015). For example, in *db/db* mice 4 weeks of treatment with the triagonist prevented excessive weight gain and reduced glycemic excursions during an intraperitoneal GTT to a significantly greater extent than that of treatment with the dual GLP-1/GIP receptor agonist. The triagonist furthermore preserved islet architecture better than did the coagonist (Finan et al. 2015). However, whether triple agonists outperform twincretins in clinical trials remains to be seen.

3.5 SGLT2 Inhibitors

Sodium glucose transporter-2 (SGLT2) inhibitors are a relatively new class of antihyperglycemic agents. They lower blood glucose levels independently of insulin action (and thus independent of beta-cell function) by inhibiting the reabsorption of glucose from the urine in the proximal renal tubules, thus facilitating its excretion (Dhillon 2019). Since their action is independent of beta-cell function, they may be

particularly useful in individuals with more advanced T2DM. Their unique mechanism of action furthermore allows for the combination of SGLT2 inhibitors with other classes of antihyperglycemic agents and minimal risk of hypoglycemia. Canagliflozin was the first SGLT2 inhibitor approved for the treatment of T2DM in various countries worldwide. Numerous multicenter trials with SGLT2 inhibitors as monotherapy or combination therapy have consistently confirmed the antihyperglycemic efficacy of SGLT2 inhibition as well as its efficacy in reducing body weight and blood pressure in a broad spectrum of patients with T2DM, including individuals with pre-existing CVD (Dhillon 2019). SGLT2 inhibitors are generally well tolerated and have a low risk of hypoglycemia, but have been associated with rare cases of diabetic ketoacidosis as well as more frequent urogenital infections (Jabbour et al. 2018a). A recent meta-analysis of three large CV outcome trials demonstrated robust benefits of SGLT2 inhibition on reducing hospitalization for heart failure and progression of renal disease regardless of pre-existing CVD. Moderate benefits have furthermore been observed on MACE, particularly in those individuals with established CVD (Zelniker et al. 2019).

3.5.1 Effects on Beta-Cell Function and Mass in Preclinical Models

SGLT2 inhibitors have been shown to preserve beta-cell mass and function in experimental models of diabetes (Cheng et al. 2016; Daems et al. 2019; Jurczak et al. 2011, 2018; Kimura et al. 2018; Liang et al. 2012; Macdonald et al. 2010; Okauchi et al. 2016; Shimo et al. 2015; Shirakawa et al. 2020). The improvements in beta-cell function observed are believed to be secondary to improved glycemic control (reversal of glucotoxicity) and weight loss, rather than being due to direct effects of SGLT2 inhibitors on beta-cells, given that beta-cells are not known to express the SGLT2 transporter (Chae et al. 2020). In *db/db* mice, *SGLT2* knockout (k.o.) or pharmacological inhibition of SGLT2 with luseogliflozin during an early stage of diabetes preserves pancreatic beta-cell function and beta-cell mass, reduces the incidence of beta-cell death and significantly improves whole-body insulin sensitivity, as assessed by hyperinsulinemic euglycemic clamp (Jurczak et al. 2011, 2018; Kimura et al. 2018). Others found SGLT2 inhibition to be associated with the stimulation of beta-cell proliferation under diabetogenic conditions in vivo (Cheng et al. 2016; Okauchi et al. 2016). For example, in *db/db* mice, luseogliflozin increases insulin biosynthesis and secretion accompanied by the increased expression of various beta-cell-specific genes, i.e. *insulin, MafA, Pdx1, and Glut2*, and increases beta-cell mass through the augmentation of beta-cell proliferation and reduction of beta-cell apoptosis (Okauchi et al. 2016).

The mechanisms underlying the effects of SGLT2 inhibition on pancreatic beta-cells have not yet been fully elucidated. In mice treated with the dual insulin/IGF-1 receptor inhibitor OSI-901, treatment with luseogliflozin for 7 days significantly increased beta-cell proliferation through the activation of the FoxM1/PLK1/CENP-A pathway. Notably, the increase in beta-cell proliferation was recapitulated in a co-culture of *Irs2* k.o. and *Insr/IR* k.o. beta-cells treated with serum from luseogliflozin and OSI-906-treated mice, indicating that humoral factors acting

independent of the IR/IGF-1R signaling pathway promote beta-cell proliferation upon SGLT2 inhibition (Shirakawa et al. 2020).

Others have suggested that SGLT2 inhibition might improve the sensitivity of beta-cells to incretins. In rodent models of diabetes, the SGLT2 inhibitor phlorizin restored the expression of the GLP1- and GIP receptors in pancreatic islets, which was found to be downregulated by chronic hyperglycemia (Asahara and Ogawa 2019; Xu et al. 1999).

Wei et al. were the first to show direct effects of SGLT2 inhibitors on pancreatic endocrine cells (Wei et al. 2020). Precisely, in two rodent models of T2DM, 6 weeks of treatment with the SGLT2 inhibitor dapagliflozin increased beta-cell number through beta-cell self-replication, alpha-to-beta-cell transdifferentiation, and duct-derived beta-cell neogenesis. In *db/db* mice, dapagliflozin treatment almost quadrupled the number of proliferating beta-cells. Importantly, dapagliflozin exerted direct effects on pancreatic endocrine cell phenotype conversion in cultured primary rodent islets and in the mouse alpha-cell line αTC1.9. Precisely, dapagliflozin upregulated the expression of pancreatic endocrine progenitor and beta-cell specific markers, including *Pdx1, Ngn-3, and Pcsk1*, and increased the GLP-1 content and secretion in αTC1.9 cells. *Pcsk1* encodes the prohormone convertase 1/3, an important enzyme for processing proglucagon to GLP-1. The dapagliflozin-induced upregulation of PDX1 expression was attenuated by the GLP-1R antagonist exendin 9–39. The authors thus concluded that the effects of dapagliflozin on pancreatic endocrine cell phenotype conversion might be partially mediated by GLP-1 secreted from alpha-cells. In support of this notion, plasma GLP-1 levels were higher in dapagliflozin treated *db/db* mice compared to control *db/db* mice (Wei et al. 2020). Notably, it has previously been shown that SGLT2 is expressed on glucagon-secreting alpha-cells of human pancreatic islets (Bonner et al. 2015). Consistently, Wei et al. showed that almost all of the alpha-cells expressed SGLT2, as did the alpha-cell line αTC1.9, while beta-cells did not (Wei et al. 2020).

3.5.2 Effects on Glycemic Control and Beta-Cell Function in Individuals with T2DM

Although SGLT2 inhibitors do not exert direct effects on beta-cells, they have been shown to enhance beta-cell function under fasting and stimulated conditions and to improve insulin sensitivity in human individuals with T2DM (Ferrannini et al. 2014; Merovci et al. 2015; Rosenstock et al. 2012; Schernthaner et al. 2013; Stenlöf et al. 2013; Wilding et al. 2013). For example, 12 weeks of canagliflozin treatment as add-on to metformin in individuals with T2DM led to dose-related significant improvements in beta-cell function compared to baseline, as indirectly assessed by HOMA2-%B, a mathematical model for the assessment of beta-cell function calculated on the basis of fasting levels for plasma glucose and insulin. Notably, numerically greater increases were found with canagliflozin compared to sitagliptin (Rosenstock et al. 2012). Similar results were obtained after prolonged treatment (Schernthaner et al. 2013; Stenlöf et al. 2013; Wilding et al. 2013). For example, after 52 weeks of treatment, a greater increase from baseline in HOMA2-%B was observed with canagliflozin compared to sitagliptin in individuals with T2DM

treated with metformin and sulfonylurea (least squares (LS) mean change compared to baseline was 21.6 for canagliflozin and 9.0 for sitagliptin, respectively) (Schernthaner et al. 2013). However, both groups showed similar postmeal improvement in indices of beta-cell function assessed during an MMTT (Schernthaner et al. 2013). Notably, the improvements in beta-cell function were associated with sustained improvements in HbA1c, FPG, body weight, and systolic blood pressure, and greater reductions were observed with canagliflozin compared to sitagliptin (mean reduction in HbA1c was 1.03% and 0.66% with canagliflozin and sitagliptin, respectively) (Schernthaner et al. 2013). These results are further supported by the work of Polidori et al., who analyzed data from three phase 3 studies, including the trial reported by Schernthaner et al., to assess measures of beta-cell function in a subset of individuals given an MMTT at baseline and study endpoint. They revealed that sustained treatment with canagliflozin for 6–12 months improves fasting and postprandial measures of beta-cell function, as shown by an upward shift and a steeper slope for the relationship between insulin secretion response and plasma glucose (Polidori et al. 2014).

Even short-term treatment with SGLT2 inhibitors has been shown to be associated with improvements in beta-cell function and insulin sensitivity (Al Jobori et al. 2018; Ferrannini et al. 2014). In individuals with T2DM, enhancing glycosuria with a single dose of the SGLT2-inhibitor empagliflozin improved beta-cell function and increased insulin-mediated glucose disposal in response to a meal (Ferrannini et al. 2014). It has therefore been assumed that lowering glucose levels by SGLT2 inhibitors can rapidly relieve the detrimental effects of hyperglycemia on beta-cell function. In Japanese individuals with T2DM, 4 weeks of treatment with the SGLT2 inhibitor ipragliflozin significantly improved beta-cell function, i.e. the disposition index (DI) assessed during an OGTT compared to baseline. After 1 week off-drug, the DI had deteriorated, but remained significantly higher compared to baseline (Takahara et al. 2015). However, it remains to be seen whether long-term SGLT2 inhibition exerts durable effects on beta-cell function and mass. Notably, in a cohort of individuals with familial renal glucosuria, caused by mutations in the SLC5A2 gene coding for SGLT2, there was no evidence of a protective effect of SGLT2 deletion with respect to changes in glucose tolerance during 30 years of follow-up (Ottosson-Laakso et al. 2016).

4 Bariatric/Metabolic Surgery

Bariatric surgery is highly effective in reducing body weight and provides substantial improvements in obesity-related comorbidities. Several studies revealed that among obese individuals with T2DM, bariatric surgery has a greater effect on diabetes remission than ILS or medical intervention (Ikramuddin et al. 2013; Mingrone et al. 2012; Schauer et al. 2017; Sjöström et al. 2014). For example, in the Swedish Obese Subjects (SOS) study, a prospective cohort study, 343 obese individuals with T2DM who underwent bariatric surgery were compared to 260 matched controls receiving standard of care. At 2 years, diabetes remission

rate, defined as FPG below 110 mg/dl and no diabetes medication, was 16% and 72% for controls and surgically treated individuals, respectively. At 15 years, the number of individuals in diabetes remission had decreased in both groups, but remained significantly higher in individuals that received bariatric surgery (30% and 7% respectively) (Fig. 1) (Sjöström et al. 2014). For more detailed information on the role of bariatric surgery in diabetes management, please refer Part III chapter "Bariatric surgery."

5 Conclusion and Outlook

The approaches to maintain or restore beta-cell function and beta-cell mass are manifold. What can be learned from the many preclinical studies and human trials is that early intervention is (in most cases) crucial to maximize the effect on beta-cell recovery. Conventional medications for the management of T2DM are largely insufficient to modify diabetes progression (Fig. 1). In the future, a more refined assessment of disease risk or disease subtype will enable more targeted therapies to prevent or treat diabetes (precision medicine). For example, several efforts have been made to refine current diabetes classifications, including data-driven cluster analysis, mainly based on immunological and metabolic parameters, to better predict specific outcomes and to enable individualized treatment regimens (Ahlqvist et al. 2018). During the last decade, genome-wide association studies have furthermore become increasingly popular to identify associations between distinct genetic variants and diabetes risk. One major focus of current research lies in exploring the value of polygenic scores, not only to predict diabetes risk, but also to refine the diagnosis of diabetes subtypes, optimize therapeutic strategies, and predict disease progression as well as the risk of diabetic long-term complications (Udler et al. 2019). Furthermore, the search for rare variants has been proposed as an alternative strategy to identify novel targets for future interventions (Salunkhe et al. 2018).

In recent years, microRNAs (miRNAs) have emerged as potential novel targets for the treatment of diabetes. miRNAs are small non-protein coding RNAs that can regulate gene expression at the posttranscriptional level by binding to potentially hundreds of mRNAs to induce their degradation or inhibition. Several miRNAs have been shown to be involved in the regulation of beta-cell function and survival as well as their differentiation and proliferation (Regazzi 2018). Alterations in the expression profiles of these miRNAs are associated with beta-cell dysfunction and death and the development of diabetes (Belgardt et al. 2015; Eliasson and Regazzi 2020). Notably, in mice, correcting the level of misexpressed miRNAs improves beta-cell function and glucose homeostasis, thus making miRNAs interesting therapeutic targets for future interventions. Circulating plasma miRNA profiles have furthermore been suggested as novel biomarkers as they are deregulated years before diabetes onset (Jiménez-Lucena et al. 2018).

Our group is investigating the role of pancreatic N-methyl-D-aspartate receptors (NMDARs), particularly in view of their effects on the regulation of beta-cell function and survival. NMDA receptors are ligand- and voltage-dependent

heterotetrameric cation channels that are widely expressed in the CNS, but are also found across a wide spectrum of non-neuronal cells, including pancreatic beta-cells. The physiological properties of central NMDARs are manifold, given that they considerably differ in terms of their regional and developmental expression, subcellular localization (e.g., synaptic or extrasynaptic), subunit composition, and interacting partners (Hansen et al. 2017). They are critically involved in normal brain functions, but are also implicated in the pathophysiology of frequent neurological and neuropsychiatric disorders such as ischemic stroke, Alzheimer's disease, and schizophrenia (Hansen et al. 2017). Although it has long been known that NMDA receptors and glutamate transporters are also expressed in pancreatic endocrine cells, and that glutamate affects the function and survival of pancreatic islets through the activation of intracellular and extracellular signaling pathways, little has been known about the role of pancreatic NMDAR in beta-cell function, and diabetes in particular (Otter and Lammert 2016). In recent years, we therefore systematically investigated NMDARs for their effects on insulin secretion, glucose homeostasis, and islet cell survival. We demonstrated that genetic deletion of pancreatic NMDARs or pharmacologic inhibition of NMDARs with either MK-801 or the over-the-counter antitussive drug dextromethorphan (DXM) increases glucose-stimulated insulin secretion from isolated mouse and human pancreatic islets and improves glucose tolerance in mice (Marquard et al. 2015). Notably, in individuals with T2DM, a single dose of DXM was sufficient to significantly enhance the blood glucose-lowering effect of the DPP4-inhibitor sitagliptin during an OGTT (Marquard et al. 2016). We also provided evidence that NMDAR antagonists are beneficial for mouse and human islet cell survival. Precisely, in *db/db* mice, continuous treatment with DXM increased islet insulin content as well as alpha- and beta-cell areas and reduced the rate of apoptosis within pancreatic islets. Furthermore, mouse and human pancreatic islets were protected from cytokine-mediated islet cell death when treated with NMDAR antagonists (Marquard et al. 2015).

Collectively these findings indicate that NMDAR antagonists have the potential to be developed as a novel class of antidiabetic drugs. Importantly, as reviewed previously (Welters et al. 2017), preclinical and clinical data indicate that DXM has angioprotective properties. In rodent models of cardiovascular diseases, DXM decreases the severity of atherosclerotic lesions and neointima formation, reduces blood pressure, and improves aortic endothelial function (Liu et al. 2009; Wu et al. 2012). In humans, it has been demonstrated that 6 months of DXM treatment enhances endothelial function, as evidenced by increases in flow-mediated dilation of the brachial artery, while the inflammatory and oxidative status were improved (Liu et al. 2008). More recently it has been shown that 12 weeks of DXM improves blood pressure control in those individuals with hypertension that fail on monotherapy with the calcium channel blocker amlodipine alone (Yin et al. 2016). Likewise, there is strong evidence from in vitro and in vivo *data* that NMDR-mediated pathways are involved in the development of diabetic nephropathy and retinopathy, and that pharmacological inhibition of renal and retinal NMDAR confers protection against glomerular and neuroretinal dysfunction under diabetogenic conditions in vivo (Welters et al. 2017). Clinical data furthermore indicate that

DXM reduces neuropathic pain, including in those individuals with diabetic neuropathy (Callaghan et al. 2012).

The broad range of effects mediated by peripheral NMDARs renders them attractive targets for the prevention and treatment of human T2DM and its long-term complications. It should however be noted that NMDAR are also involved in the activation and polarization of human T-cell responses. Isolated human $CD4^+$ T-cells express functional NMDA receptors, and their expression is markedly increased upon T-cell activation (Orihara et al. 2018). Furthermore, the activation of NMDARs has been shown to differentially affect human T-cell subsets in terms of cytokine expression, proliferation, and cell survival, thus contributing to the modulation of immune responses (Orihara et al. 2018). Since these processes are also critically involved in the emergence of autoimmune responses, modulating NMDA receptor signaling may potentially affect the course of autoimmune disorders, such as type 1 diabetes, besides being beneficial for individuals with T2DM.

In fact, there is considerable evidence that NMDAR antagonists elicit positive immunomodulatory effects. DXM has been shown to inhibit LPS-induced functional maturation of mouse and human dendritic cells (DC), and dose-dependently reduced the production of proinflammatory cytokines (e.g., TNF-alpha, IL-6 and IL-12), chemokines (e.g., MCP-1, MIP-1 alpha, and RANTES) and oxidative stress from murine DCs. DXM furthermore impaired the ability of LPS-induced murine DCs to activate antigen-specific T-cell responses, as evidenced by decreased T-cell proliferation and IFN-gamma secretion in mixed leukocyte cultures (Chen et al. 2013). In a mouse model of rheumatoid arthritis (RA), an inflammatory autoimmune disease of the joints, DXM attenuated arthritis symptoms and reduced serum levels of proinflammatory cytokines, including TNF-alpha. Notably, in human individuals with RA, 6 months of DXM treatment (120 mg once daily) as add-on to disease-modifying-antirheumatic drugs significantly reduced the serum levels of TNF-alpha and IL-6 as compared to baseline and to non-DXM-treated individuals. Numerically greater decreases were furthermore observed for serum levels of IFN-gamma and IL-17A levels in DXM-treated individuals compared to those without DXM (Chen et al. 2017).

In view of these data, it is conceivable to think that NMDAR antagonist also positively affect autoimmune responses in individuals with T1DM, and this hypothesis is currently under investigation in preclinical setting (Wörmeyer, Lammert, Welters, unpublished data). Thus, NMDAR antagonists seem to have a broad spectrum of activity in terms of beta-cell function and survival, diabetic long-term complications, and immunomodulation. However, additional studies and long-term clinical trials are required to validate the effects of NMDAR inhibition on pancreatic beta-cells, glycemic control, and diabetic long-term complications in humans with diabetes.

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Precision Medicine and Obesity

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Abstract

Obesity is a chronic, relapsing, and multifactorial disease, with a rising prevalence and an associated high economic burden. Achieving successful and sustained weight loss outcomes with current interventions is challenging. This is due, at least in part, to the disease's heterogenous pathophysiology that is yet to be completely understood. Technological advances and greater capabilities for the extraction and storage of information have facilitated the application of precision medicine. Several precision medicine initiatives have been proposed to improve obesity outcomes. Most of these initiatives are based on -omics technologies. Although the data generated from these technologies have led to developing hypotheses that may explain the underpinnings of obesity, their applicability to the clinical practice is yet to be determined. There are other initiatives that have identified quantitative or qualitative physiologic traits that can be targeted and that could have a more immediate clinical impact. This review aims to provide a perspective of current initiatives for precision medicine for obesity.

Keywords

Obesity · Phenotype · Precision medicine

1 Introduction

Over time, the practice of medicine has shifted from an approach of disease treatment based on the physician's subjective interpretation of signs and symptoms (intuition medicine) to an evidence-based approach guided by clinical trials, the most reliable source of scientific information. As the practice of medicine continues to evolve, we are now witnessing the application of personalized medical interventions that are selected based on algorithms containing quantifiable patient data in the form of genetics, genomics, epigenetics, metabolomics, and proteomics, among others (Cass 2014; Hood and Flores 2012; Toledo et al. 2012). This has been referred to as precision medicine (Fig. 1).

In 2011, the National Research Council defined precision medicine as any medical intervention targeted to population subgroups categorized on common genetic patterns, lifestyles, drug responses, and/or environmental and cultural factors (Hopp et al. 2018). The development of precision medicine has allowed a better understanding of a disease's pathophysiology that has been essential for developing personalized interventions. Precision medicine has also led to the development of tools that can predict a disease's natural history as well as the response to interventions (Hood and Flores 2012). It is estimated that this new therapeutic model could help lessen the economic healthcare burden of chronic diseases by 50% (Hood and Flores 2012).

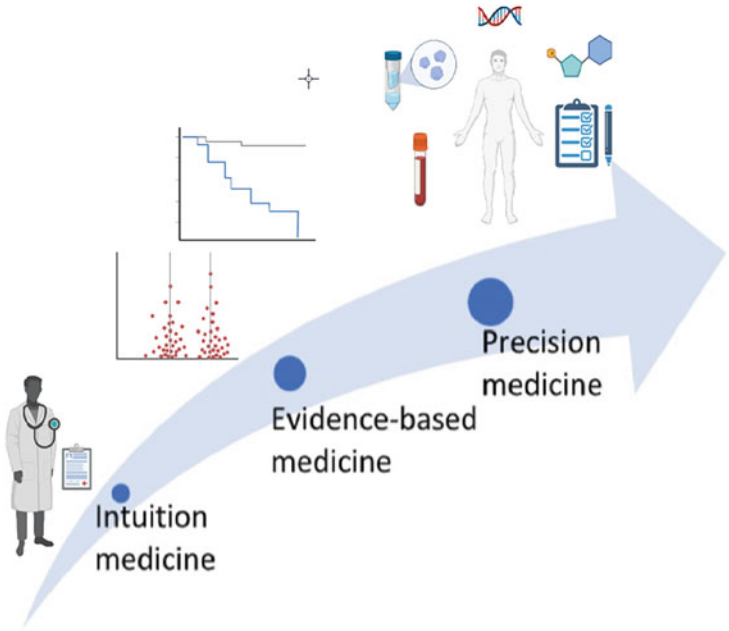

Fig. 1 Progression of the practice of medicine through time

Obesity is a chronic, relapsing, multifactorial disease with a projected prevalence of 50% by 2030 and a high economic burden (Cawley and Meyerhoefer 2012; Colditz 1992; Collaborators et al. 2017; Dobbs et al. 2014; Waters and Graf 2018; Hales et al. 2017, 2020; Heymsfield and Wadden 2017; People 2020; Trogdon et al. 2008; Wang et al. 2011; Ward et al. 2019; Withrow and Alter 2011). Obesity treatment is challenging because of the redundant and adaptive pathways to preserve energy. Consequently, successfully sustained weight loss outcomes with current treatment paradigms remain a challenge to achieve in clinical practice (Heymsfield and Wadden 2017; Loos and Janssens 2017; MacLean et al. 2017). The heterogeneity of obesity is particularly apparent in the highly variable response to current weight loss interventions, including diets, anti-obesity medications, devices, and surgery (Heymsfield and Wadden 2017; Khera et al. 2016). The current standard of care in the treatment of obesity relies on providers selecting a weight loss intervention based on the patient's BMI, provider's and/or the patient's preference, comorbidities, possible medication interactions when medications are selected, the risk of potential adverse events, and/or insurance coverage (Acosta et al. 2017; Gadde et al. 2018; Heymsfield and Wadden 2017; Igel et al. 2017).

The standard of care to treat obesity is clearly not working as we continue to see an increase in the prevalence of obesity worldwide. As a result of this trend and with the advent of precision medicine, research efforts in the obesity field are now focusing on understanding the heterogeneity of human obesity and identifying predictors of response to weight loss interventions that can be targeted to enhance weight loss outcomes and facilitate weight loss maintenance (Curioni and Lourenco 2005; MacLean et al. 2015; Piché et al. 2020).

This review aims to describe the current precision medicine for obesity initiatives, emphasizing those that can have an immediate impact in clinical practice.

2 Obesity Phenotypes Based on Pathophysiologic Traits

2.1 Characterization of Obesity Phenotypes

One of the initiatives in precision medicine for obesity has focused on targeting specific pathophysiologic processes that lead to energy balance dysregulation and that result in excessive storage of calories in the form of fat (Acosta et al. 2014). Energy balance depends on energy expenditure and energy intake, two tightly regulated processes controlled by humoral and neuronal signals in response to internal and external cues (van der Klaauw and Farooqi 2015). On the one hand, the key determinants of energy expenditure are resting energy expenditure (REE), non-exercise physical activity, the thermogenic effect of food, and exercise (Hopkins and Blundell 2016). On the other hand, the key determinants of energy intake are the homeostatic and hedonic drives to eat (Blundell and Finlayson 2004). The homeostatic drive to eat, mainly controlled by the brain-gut axis, involves hunger (desire to eat), satiation (the physiologic process that promotes meal termination), and satiety (post-prandial events that determine the timing for the next meal) (Acosta et al. 2015a, b; Camilleri 2015; Cifuentes and Acosta 2021). The hedonic energy intake component is determined by the desire to eat to obtain pleasure in the absence of an energy deficit (Avena 2015; Tulloch et al. 2015).

All these aspects of energy balance can be quantified. For instance, hunger is measured using a visual analog scale after fasting and before eating a meal. Satiation is measured through ad libitum intake of food or drinks while monitoring calories or volume consumed before fullness sensation is reached. Two validated tools are the nutrient drink test that determines gastric volumes associated with meal termination and the ad libitum meal test that determines the calories to fullness during an all-you-can-eat meal (Blundell et al. 2010; Chial et al. 2002). Satiety is measured with visual analog scales for appetite after a meal and with gastric emptying by scintigraphy (Acosta et al. 2021; Gonzalez-Izundegui et al. 2021). Hedonic eating behavior is quantified with a series of validated questionnaires which include the Hospital Anxiety and Depression Score (HADS) (Acosta et al. 2015a, b) and Three-Factor Eating Questionnaires (TFQ-21) (Karlsson et al. 2000). Furthermore, functional imaging of the brain is also used to objectively quantify neuronal activity in areas that participate in the control of homeostatic and hedonic drives of food intake (Devoto et al. 2018; Zanchi et al. 2017). Finally, energy expenditure is quantified by assessing the amount of activity via self-report or activity trackers, and by measuring resting energy expenditure by indirect calorimetry in relation to body composition measured by dual-energy X-ray absorptiometry (Cooper et al. 2009).

The ability to quantify these aspects of energy balance has led to the discovery of specific gastrointestinal and behavioral traits that differ between people with normal weight and people with obesity (Acosta et al. 2015a, b). The predominant traits in

people with obesity include a higher fasting gastric volume for both solids and liquids, accelerated gastric emptying for solids and liquids, a higher volume and/or calories to reach fullness, higher anxiety and depression scores, and lower body image satisfaction (Acosta et al. 2015a, b). Initial observations of these traits showed that adults younger than 35 years old with obesity and accelerated gastric emptying gained more weight compared to those with normal gastric emptying when followed prospectively for 4 years (Pajot et al. 2020). However, more studies are needed to understand whether these traits are cause or consequence of obesity.

In the meantime, these traits have been used to classify obesity based on pathophysiological and behavioral phenotypes. The classification was initially based on a supervised principal component analysis (Acosta et al. 2015a, b), followed by a simplified stratification defined by arbitrary cut-offs based on inter-quantile ranges (Acosta et al. 2021). To date, four phenotypes have been identified: abnormal satiation associated with abnormal hypothalamic brain perfusion; abnormal satiety associated with intestinal enteroendocrine cell dysfunction; abnormal emotional eating characterized by uncontrolled hedonic eating and associated with abnormal nucleus accumbens perfusion, and abnormal resting energy expenditure associated with decreased REE by indirect calorimetry (Acosta et al. 2015a, b, 2021).

The differences of each phenotype compared to other phenotypes were as follows: participants with abnormal satiation phenotype consumed 62% more calories before reaching fullness; participants with abnormal emotional eating phenotype reported 2.8 times higher levels of anxiety; participants with abnormal satiety phenotype emptied the stomach contents 31% faster; and participants with abnormal resting energy expenditure phenotype had 12% lower predicted REE (Acosta et al. 2021). Abnormal satiation and abnormal satiety phenotypes are the most prevalent obesity phenotypes (Fig. 2). While multiple obesity phenotypes can coexist in the same individual, 15% of patients do not meet the criteria for any of the obesity phenotypes identified to date (Acosta et al. 2021).

2.2 Obesity Phenotyping as a Tool for Prediction and Treatment for Weight Loss

Post-hoc analyses of pilot studies using anti-obesity medications (AOM) and bariatric endoscopic devices have shown that these obesity phenotypes predict weight loss response to these interventions (Abu Dayyeh et al. 2017; Acosta et al. 2015a, b; Gomez et al. 2016; Halawi et al. 2017; Lopez-Nava et al. 2020; Sivamaruthi et al. 2019; Vargas et al. 2019, 2020). One of these studies, for instance, showed that phentermine-topiramate extended-release (ER) works better for the abnormal satiation phenotype (Acosta et al. 2015a, b). In this 14-day trial of 24 patients, phentermine-topiramate ER was associated with reduced food intake at an ad libitum meal (mean Δ 260 kcal, $p = 0.03$) and delayed gastric emptying of solids (mean Δ gastric emptying $T_{½}$ 19 min, $p = 0.06$). Patients on phentermine-topiramate ER had a greater mean weight loss of 1.4 kg compared to placebo. Abnormal satiation at

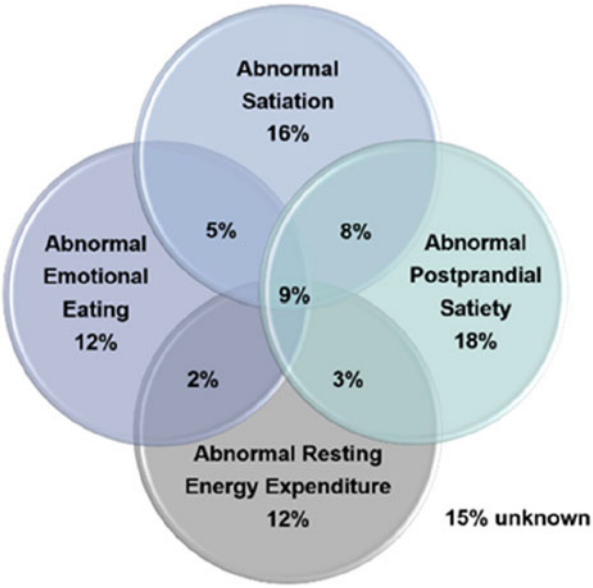

Fig. 2 Distribution of obesity phenotypes in 450 patients with obesity (Acosta et al. 2021)

baseline was associated with greater total weight loss on phentermine-topiramate ER.

Studies have also investigated the effect of glucagon-like peptide-1 (GLP-1) receptor agonists on the phenotypes. For instance, exenatide, 5 μg subcutaneous twice daily for 30 days, significantly slowed gastric emptying of solids ($p < 0.001$) and reduced calorie intake at an ad libitum meal by an average of 130 kcal compared to placebo. The average weight loss was 1.3 kg for the exenatide group and 0.5 kg for the placebo group (Acosta et al. 2015a, b). Similarly, liraglutide administered daily for 16 weeks delayed gastric emptying at 5 ($p < 0.0001$) and 16 ($p = 0.03$) weeks ($p = 0.03$) compared to placebo. Liraglutide also resulted in higher weight loss at 16 weeks (6.1 ± 2.8 kg (SD) compared to 2.2 ± 5 kg control group, $p < 0.01$). At 5 and 16 weeks, gastric emptying of solids correlated with differences in weight loss on liraglutide ($p < 0.02$) (Halawi et al. 2017). Thus, having an accelerated gastric emptying, characteristic of the abnormal satiety phenotype, was a predictor for a better weight loss response when GLP-1 receptor agonists are used.

Based on these data and the understanding of the mechanisms of action of AOM, it has been hypothesized that AOM can be used to specifically target obesity phenotypes (Fig. 3). For instance, as the abnormal satiation phenotype is characterized by a higher caloric requirement at each meal to reach fullness, these patients might benefit the most from phentermine-topiramate ER, a medication that modulates the adrenergic and GABAergic pathways resulting in satiation (Acosta et al. 2017; Igel et al. 2017). Similarly, as the abnormal satiety phenotype is characterized by accelerated gastric emptying and increased post-prandial hunger, these patients might benefit the most from GLP-1 receptor agonists, medications that delay gastric emptying and reduce inter-meal hunger (Acosta et al. 2015a, b; Halawi

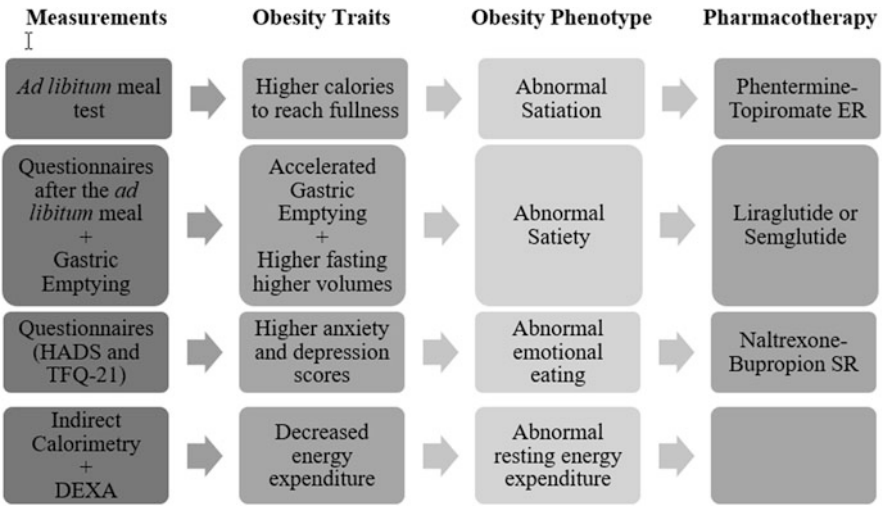

Fig. 3 Flow chart describing the measurements used for each obesity trait that led to the discovery of the obesity phenotypes, in addition to its specific pharmacotherapy

et al. 2017; Kadouh et al. 2020; Tchang et al. 2000; van Can et al. 2014). Finally, as the abnormal emotional hunger phenotype is characterized by negative mood eating and reward-seeking behaviors in relation to negative and positive emotions, these patients might benefit the most from naltrexone/bupropion sustained-release, a medication that modulates the dopamine and opioid pathways (Acosta et al. 2017; Igel et al. 2017). No AOM is known to increase metabolism, and therefore there is currently no pharmacologic therapy that could be used for the abnormal resting energy expenditure phenotype.

Based on this working hypothesis, AOMs were used to target specific phenotypes in a large real-world pragmatic study. The data revealed that after 12 months of phenotype-guided anti-obesity medication, the proportion of patients who lost >10% at 12 months was 79% in the phenotype-guided group compared to 34% in the non-phenotype-guided treatment group (Fig. 4a). This approach decreased the therapy failure rate (<5% total body weight loss). The phenotype-guided approach was associated with 1.75-fold greater weight loss after 12 months, with a mean weight loss of 15.9% compared to 9.0% in the non-phenotype-guided group (Fig. 4b) (Acosta et al. 2021).

This approach has several limitations. Studies to date are unblinded, and therefore the superiority of the phenotype-guided approach could be partially attributed to performance bias resulting from participants and providers knowing the participants' phenotype. Second, the generalizability may be limited due to the modest sample size and the lack of diversity of the cohorts. Third, the physiologic studies required to identify the obesity phenotype(s) to which a participant belongs can only be performed at large academic or research institutions. Fourth, some patients do not meet the criteria for currently identified phenotypes. For this model to become more

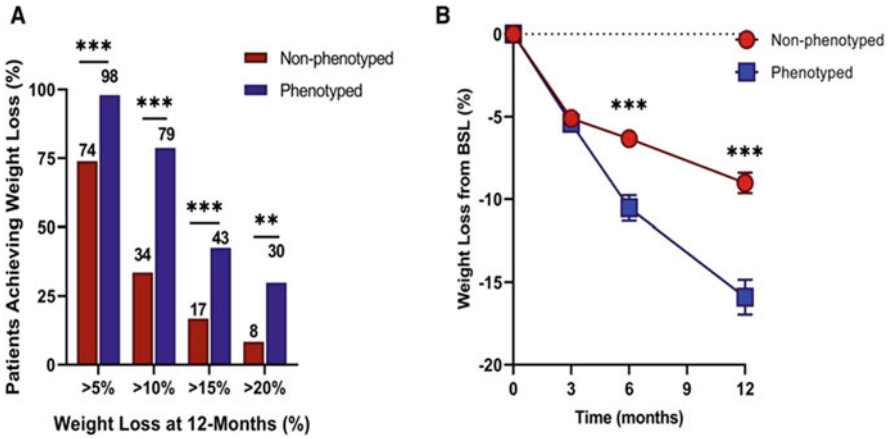

Fig. 4 Phenotype-guided medications for obesity management improves weight loss outcomes. (**a**) Proportion of patients achieving >5%, >10%, >15%, and >20% weight loss based on phenotype (blue) and non-phenotype (red) guided anti-obesity pharmacotherapy. (**b**) Weight loss percentage from baseline of patients treated with phenotype (blue) vs. non-phenotype (red) guided anti-obesity pharmacotherapy

applicable in clinical practice, first, it is essential to study the phenotype-guided approach in prospective, randomized, large-scale controlled trials in a more diverse population. Second, there is a critical need to simplify the methods to identify obesity phenotypes. Third, more studies are needed to determine whether other obesity phenotypes (i.e., the microbiota profile, leptin levels, insulin levels, etc.) should be considered and included in the stratification and management of obesity.

3 Psycho-Behavioral Phenotyping of Obesity: The Six-Factor Questionnaire (6FQ)

The six-factor questionnaire (6FQ) is considered a psycho-behavioral phenotyping. It was developed to make obesity counseling and management in the primary care setting more effective, efficient, and compassionate (Kushner et al. 2016; Kushner and Hammond 2021).

This 27-item questionnaire assesses behavioral, cognitive, and affective lifestyle factors related to weight gain (Kushner et al. 2016; Kushner and Hammond 2021). Six factors have been recognized: convenient diner, easily enticed eater, exercise struggler, fast pacer, self-critic, and all-or-nothing doer. These six factors represent unhealthy lifestyle patterns associated with diet, physical activity, cognition, and self-perception. There is a positive and strong correlation between factor scores and BMI. For instance, for every unit increase in average score in the exercise struggler factor, BMI increases by 3.85 kg/m^2 (Kushner and Hammond 2021).

The contribution of this tool to precision medicine for obesity relies on its ability to improve patient communication, shared-decision making, health provider-patient

relationships, and health promotion. Also, this tool provides information about what the patient does, thinks, and believes. This psycho-behavioral segmentation could potentially allow for a more personalized approach to managing obesity.

Its clinical applicability relies on its feasibility. It is a short, self-administered questionnaire that a patient can complete before a doctor's visit. Furthermore, it can be easily scored and interpreted by any health care provider. All these characteristics allow for a more efficient and effective counseling session for weight management. However, the applicability of this tool has limitations. First, although it has been validated in two cohorts, most participants were White and females, making it less generalizable (Kushner et al. 2016). Second, there is no data to date supporting that this approach leads to better weight loss outcomes compared to the current standard of care.

4 The Role of Post-Prandial Glucose Metabolism on Weight Management: Glycemic Dips

Post-prandial satiety plays an important role in food intake regulation and has become an area of research interest for the treatment of obesity. Various factors influence hunger sensation after a meal, as well as the time and size of the subsequent meal. Gastric distention, gastrointestinal peptides, and plasma metabolites are just a few (Wyatt et al. 2021). Among metabolites, glucose has gained particular attention in recent years. The glycemic load of a meal triggers the release of insulin. When insulin binds its receptors on the hypothalamus, it stimulates the release of anorexigenic peptides, thereby inhibiting appetite. A disturbance in this system can affect post-prandial appetite and therefore impact energy intake.

Obesity is associated with insulin resistance that diminishes the glucose's ability to reach its target organs, including the brain. As a result, insulin resistance may result in a neuroglycopenic state that activates the drive of feeding, thereby affecting post-prandial satiation (Wyatt et al. 2021). Studies have demonstrated that the 2–3-h post-prandial glucose dip is strongly associated with post-prandial satiation and, therefore, can be used as a predictor tool of energy intake in a subsequent meal. This predictive tool has been studied in patients with obesity, in whom a lower glucose nadir preceding a meal has a high predicted value for hunger and subsequent energy intake (Kim et al. 2019). The main factor that has been shown to affect glucose dips and increase the risk of weight gain is high glycemic loads during meals (Bao et al. 2009; Wyatt et al. 2021).

Glycemic dips have now been used to offer personalized dietary advice to patients with obesity. Its clinical application has been facilitated by the widespread accessibility to technology devices such as continuous glucose monitors, wearable devices, and tracking apps.

5 Pharmacotherapy for Monogenic Mutations Associated with Obesity

Genetic mutations account for 5% of all cases of obesity (Saeed et al. 2018). Most of these cases are polygenic in origin, and there are no current clinical therapies for this type of mutations. Proopiomelanocortin (*POMC*) and leptin receptor (*LEPR*) deficiency are obesity monogenic disorders characterized by biallelic variants in *POMC* or *PCSK1*, and *LEPR*, respectively (Clément et al. 2020a). Because these are rare disorders, providers generally do not consider these conditions in their differential diagnosis for obesity and therefore are underdiagnosed (Huvenne et al. 2016). Precision medicine has shown to be beneficial and to have a clinical impact on patients who carry these monogenic mutations.

For instance, recombinant leptin has been used successfully to treat obesity resulting from congenital leptin deficiency (Farooqi et al. 2002). Similarly, patients with POMC deficiency or LEPR deficiency have had promising weight loss outcomes with setmelanotide, a melanocortin-4 receptor agonist, the receptor through which POMC signals (Clément et al. 2020b; Collet et al. 2017; Farooqi et al. 2002; Haws et al. 2017). Setmelanotide has been approved by the Food and Drug Administration for *PSCK1*, *LEPR*, and *POMC* homozygous mutations. These are some of the genes that participate in POMC signaling. The use of setmelanotide for these mutations has been shown to decrease body weight and hunger, but with variable results (Clément et al. 2018). Studies have shown that the percentage of patients achieving 10% of total body weight loss after 1 year of setmelanotide therapy was 80% for patients with a *POMC* mutation, and 45% for those with an *LEPR* mutation. The mean total body weight loss percentage at 1 year was 25.6% for *POMC* mutation carriers and 12.5% for *LEPR* mutation carriers (Clément et al. 2020b). The fact that some patients with common obesity respond to these therapies supports the existence of single-nucleotide polymorphisms (SNPs) with a shared mechanistic gene of action (Collet et al. 2017; Roth et al. 2008). Identifying these SNPs could offer an additional therapeutic modality for at least a subgroup of patients with common obesity that may benefit from these two treatments.

6 Other Precision Medicine for Obesity Initiatives

Additional precision medicine initiatives for obesity arise from the concept that genetics and environmental factors play an essential role in the development and progression of obesity. In recent years, and with the advent of ‘omics technologies, biological variants associated with obesity such as transcripts, proteins, metabolites, microbiota, and epigenetic markers, among others, have been identified (Fig. 5). Although some of these biological variants participate in the pathophysiology of obesity, their applicability in clinical practice remains to be ascertained. Here, we summarize the data on these initiatives.

Fig. 5 Multi-omics

1. Nowadays, Genome-Wide Association (GWAS) has identified more than 140 genes associated with obesity (Fall et al. 2017). Although the genes related to leptin and melanocortin signaling and those related to chromosome 15 (Prader Willi syndrome) have a large effect size on BMI, the rest of these genes have a negligible effect size on obesity-related anthropometric variables. More investigation is needed to understand the role of these genes on the pathophysiology of obesity for targeted therapies to be developed (Fall et al. 2017; Locke et al. 2015).
2. Even though technological advancements in genetics have led to the discovery of more than 300 SNPs, their effect on BMI is modest (20%) (Locke et al. 2015; Yang et al. 2015). Currently, the clinical applicability of SNPs is restricted to those who are treated for monogenic obesity syndromes, as described above (Collet et al. 2017). Before SNPs become an important tool for individualizing obesity interventions, more information on their influence on gene expression is needed.
3. Epigenetic modifications due to environmental factors alter gene activity and are associated with obesity traits (Cordero et al. 2015; Hurtado and Acosta 2021). Their importance has been indirectly demonstrated through epigenome changes observed after dietary, physical activity, and/or surgical interventions (Barres et al. 2013; Milagro et al. 2011; Rönn et al. 2013). Epigenome profiling

microarrays are clinically available and inexpensive. Although there is evidence that this information could be used as a prognostic and diagnostic tool, its clinical application for precision medicine for obesity interventions is limited.

4. Pharmacogenomics, the study of individual responses to pharmaceutical compounds based on genetics, has demonstrated clinical relevance. It can predict genetic variants that would affect the susceptibility and response of a weight loss medication (Acosta et al. 2021). The widespread application of this technology may be limited by the costs associated.
5. The interplay between the type of macronutrient and individual's genotype could play a role in obesity (San-Cristobal et al. 2020). Through nutrigenetics and nutrigenomics data, diets can be tailored to the specific gene variants involved in energy metabolism. There is contradictory data on whether this approach can lead to better weight loss outcomes compared to standard of care (Arkadianos et al. 2007; Gardner et al. 2018).
6. There is an established relationship between obesity and gut microbiome derangements. Studies have shown that, on the one hand, these microbiome derangements can be reversed with weight loss interventions, and on the other hand, microbiome manipulation could promote weight loss (Behrouzi et al. 2019; Palleja et al. 2016). The advancement in genome sequencing and innovative cultivation methods has allowed the discovery of new microorganisms (next-generation probiotics) with direct potential health benefits (O'Toole et al. 2017). However, the clinical use of these probiotics remains limited by data on safety, efficacy, and the costly and intricate mass production process (Brusaferro et al. 2018; Cunningham et al. 2021; Michael et al. 2020; Nicolucci et al. 2017; Sivamaruthi et al. 2019). Fecal matter transplantation has also been a focus of attention for treating obesity and other metabolic disorders, but results are variable, and therefore its clinical application remains limited (Hartstra et al. 2015; Sivamaruthi et al. 2019). Future efforts aimed at diet optimization to improve the gut microbiome could be feasible, accessible, and practical.
7. Metabolomic signatures have been identified among patients with obesity (Hurtado and Acosta 2021; Rangel-Huerta et al. 2019). Their primary clinical applicability is related to the characterization and development of biomarkers that can be used to predict outcomes to weight loss interventions (Geidenstam et al. 2017; Stroeve et al. 2016).

Table 1 summarizes common SNPs, epigenetically modified genes, SNP–diet interactions, and metabolic pathways associated with obesity and obesity traits.

7 Conclusion

The complex pathophysiology behind obesity, including the biological variants affecting the disease, has opened new opportunities for initiatives that use precision medicine as their primary approach. Precision medicine has offered a new perspective for obesity treatment. Even though technological advances and increased

Table 1 Common SNPs, epigenetically modified genes, SNP–diet interactions, and metabolic pathways associated with obesity and obesity traits

SNPs Wu et al. (2017), Goodarzi (2018), Chehadeh et al. (2020), Llanaj et al. (2020), Schlauch et al. (2020)	
Gene	Phenotype
FTO	BMI, waist circumference, fat percentage, extreme obesity
MC4R	BMI, waist circumference, extreme obesity
MC3R, SLC6A14	Obesity
POMC, NEGR1, PCSK1, GNPDA2, MAP2K5, SEC16 B	BMI
FTO, NEGR1, ADAMTS16, FUT9, TDH, NCKAP5L, FAM167A, LMO1, RTN4RL1, CABP5	BMI
PFKFB3, KLRB1, GRIN2A	Obesity
PPARγ	Waist circumference
KCTD15, SH2B1, TFAP2B	Overweight/obesity
BDNF, MC4R	Childhood obesity

Epigenetically modified genes (Rohde et al. 2019)	
Gene	Phenotype
POMC, NPY, SLC6A4, MCHR1	Overall obesity
FTO, LPL, IRS 1, TMEM18	Fat distribution
PPARG	Percentage body fat
LEP	Overall obesity, fat distribution, BMI

SNP–diet interactions (Ramos-Lopez et al. 2017)		
Gene	Diet interaction	Putative disease risk
FTO	High fat and high carbohydrate dairy products high fat	Obesity
LCT	Dairy products	
PPARG, G1PR	High fat	
TXN	Low vitamin E	Abdominal obesity
MC4R	Western dietary pattern and high saturated fatty acids	Metabolic syndrome
APOB	High fat	
TCF7L2	High saturated fatty acids	
APOC3, APOA1	Western dietary pattern	

Deregulated metabolic signatures (Newgard 2017)	
Metabolic pathway	Phenotype
Branched-chain amino acid metabolism	Obesity and insulin resistance
Androgen synthesis	Childhood obesity

SNP–environmental interactions (Mason et al. 2020)		
Gene	Environmental interaction	Putative disease risk
MC4R	Proximity to takeaway/fast-food outlets	Obesity

SNP–obesity–tumorigenesis interaction (Lan et al. 2020)		
Gene	Phenotype	Putative disease risk
FTO	Fat mass	Cancer

Adapted from Hurtado and Acosta (2021)

information storage capabilities have made it possible to understand this disease better, the clinical applicability of most of this precision medicine for obesity initiatives is limited.

Obesity phenotyping has arisen as a need for diagnostic optimization and treatment response improvement. Clinically, its capacity to rely on objective outcomes, as are its quantifiable traits, make phenotyping a well-grounded tool for clinicians to select the appropriate AOM for the right patient with the end goal of improving weight loss outcomes. Translating this precision medicine approach to a larger and more heterogenous population will undoubtedly increase its external validity and, therefore, its generalizability, which is the ultimate goal in clinical practice.

Disclosure Statement

- Daniel Sacoto and Maria Daniela Hurtado A. have nothing to disclose.
- Andres Acosta is a stockholder in Gila Therapeutics and Phenomix Sciences; he serves as a consultant for Rhythm Pharmaceuticals, General Mills.

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Targeting the Enteroendocrine System for Treatment of Obesity

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Abstract

Mimetics of the anorexigenic gut hormone glucagon-like peptide 1 (GLP-1) were originally developed as insulinotropic anti-diabetic drugs but also evoke significant weight loss, leading to their recent approval as obesity therapeutics. Co-activation of receptors for GLP-1 and other gut hormones which reduce food intake – peptide YY ($PYY_{3–36}$), cholecystokinin (CCK) and glucose-dependent insulinotropic peptide (GIP) – is now being explored clinically to enhance efficacy. An alternative approach involves pharmacologically stimulating endogenous secretion of these hormones from enteroendocrine cells (EECs) to recapitulate the metabolic consequences of bariatric surgery, where highly elevated postprandial levels of GLP-1 and $PYY_{3–36}$ are thought to contribute to improved glycaemia and weight loss.

Keywords

CCK · Diabetes · Enteroendocrine · GIP · GLP-1 · Gut hormone · Obesity · PYY

1 Introduction

Obesity is a growing global health concern, and the major modifiable risk factor for type 2 diabetes mellitus (T2DM) and cardiovascular disease. Even relatively modest weight loss (5–10%) can substantially improve insulin sensitivity, pancreatic β-cell function, inflammation, and cardiovascular risk scores (Magkos et al. 2016). Beyond lifestyle modification and poorly-tolerated drugs, the most effective weight loss strategy for common obesity has been bariatric surgery.

Several pharmacological agents targeting the gut hormone axis have recently been developed as novel obesity therapeutics, after successful use in the treatment of T2DM. Once-weekly injections of the long-acting glucagon-like peptide 1 (GLP-1) mimetic semaglutide were recently shown to promote substantial weight loss in obese subjects in a series of phase III trials, with reductions of over 15% observed in half of participants (Wilding et al. 2021). This heralds a new era for obesity therapeutics and encourages development of other medications which activate the same anorexigenic pathways, while minimising side effects. This chapter examines existing and potential approaches to target the enteroendocrine system in obesity, including drugs which directly activate receptors for GLP-1 and other gut hormones, bariatric surgery, and modulation of endogenous enteroendocrine cell (EEC) secretion.

2 Gut Hormone Mimetics: GLP1R Agonism and Beyond

2.1 GLP-1 Receptor Agonists

GLP-1 itself cannot be given therapeutically due to its extremely short half-life (1–2 min), so several injectable GLP1R agonist peptides which are partly resistant to dipeptidyl peptidase 4 (DPP4) degradation have been developed (Drucker and Nauck 2006). Since the approval of the first such GLP1R agonist exenatide in 2005, significant advances have been made to increase half-life, allowing a shift from twice-daily to once-weekly injections and oral formulations (Marso et al. 2016; Zinman et al. 2019). Prominent side effects of incretin mimetics include nausea and GI symptoms such as diarrhoea, although these are somewhat reduced in second-generation treatments and are normally manageable following dose titration (Drucker and Nauck 2006; Zinman et al. 2019).

Following phase III trials showing outstanding weight loss efficacy of 10–20% in overweight subjects, the long-acting GLP1R agonist semaglutide has recently received FDA approval for treatment of obesity (Ryan 2021). This weight loss was far greater than the 5–10% achieved with another GLP1R agonist liraglutide, which was FDA-approved for obesity in 2014 (Mehta et al. 2017). This success is likely to encourage development of next-generation anti-obesity drugs targeting the GLP-1 system, which have the dual benefit of weight loss and improved glycaemic control. GLP1R agonists also reduce the risk of cardiovascular events such as stroke and myocardial infarction (Kristensen et al. 2019), although the underlying mechanisms and the extent of GLP1R expression in the heart and blood vessels remain unclear (McLean et al. 2021).

2.2 DPP4 Inhibitors

Another class of antihyperglycaemic drugs targeting the incretin system are the orally-bioavailable DPP4 inhibitors which increase the circulating half-life of both GLP-1 and the related hormone glucose-dependent insulinotropic peptide (GIP). While DPP4 inhibitors such as sitagliptin increase glucose-stimulated insulin secretion, they do not induce significant weight loss, unlike GLP1R agonists (Drucker and Nauck 2006). DPP4 has a broad range of other substrates, including peptide YY (PYY) and neuropeptide Y, which may have implications for the overall metabolic effects of DPP4 inhibition (Mulvihill and Drucker 2014). DPP4 cleavage converts $PYY_{1–36}$ to $PYY_{3–36}$, which mediates the majority of PYY's anorexigenic effects via NPY2R (Ballantyne 2006). Indeed, the appetite-suppressive effects of $PYY_{1–36}$ are lost in rats genetically deficient in DPP4 (Unniappan et al. 2006) and activation of NPY1R is linked to increased feeding (Kanatani et al. 2000). It has therefore been proposed that reduced $PYY_{3–36}$ and increased $PYY_{1–36}$ levels following DPP4 inhibition account for the lack of weight loss (Aaboe et al. 2010). An alternative possibility, however, would be that intestinal-derived active GLP-1 levels reached under normal physiological conditions are insufficient to promote weight loss. This

is supported by findings that the DPP4 inhibitor linagliptin did not enhance anorexia in diet-induced obese (DIO) mice even in the presence of an NPY2R agonist, despite elevated active GLP-1 levels (Hansen et al. 2021), and that GLP1R inhibition did not affect food intake reduction seen following selective chemogenetic activation of distal colonic L-cells, whereas NPY2R inhibition did (Lewis et al. 2020).

2.3 GLP1R/GCGR Co-agonism

In the development of novel incretin mimetics, there is increasing interest in the use of dual or triple agonists targeting other receptors in addition to the GLP1R. The rationale is that these may combine beneficial effects on body weight, glycaemic control and insulin sensitivity while minimising side effects (Brandt et al. 2018). The co-activation of gut hormone receptors also more closely mimics the physiological response to a meal, where multiple enteroendocrine peptides are co-secreted.

Despite the glucose-elevating effects of glucagon (GCG), chronic administration increases energy expenditure, likely via central mechanisms and hepatic upregulation of fibroblast growth factor 21 (FGF21) (Habegger et al. 2013). Glucagon action in the liver also reduces hepatic lipid content and alters hepatocyte metabolism to reduce the extent of non-alcoholic steatohepatitis (NASH), a common obesity co-morbidity (Boland et al. 2020). Balanced synthetic co-agonists of the GLP1R/GCGR, which mimic the activity of oxyntomodulin, are capable of improving glycaemic control in mice and humans (Ambery et al. 2018; Henderson et al. 2016). The GLP1R/GCGR co-agonist SAR425899 induced similar weight loss to liraglutide in a phase IIb trial, while improving HbA1c and increasing β-cell responsiveness to a mixed meal tolerance test (Schiavon et al. 2021); however, this drug was discontinued in 2018 due to incidence of adverse events and disappointing efficacy (Sanofi 2018). A separate phase IIb trial in overweight T2DM patients showed the GLP1R/GCGR co-agonist cotadutide (MEDI0382) evokes slightly greater weight loss (~5%) than a higher dose of liraglutide (Nahra et al. 2021). Unlike liraglutide, cotadutide reduces hepatic fibrosis and lipid content in preclinical NASH models (Boland et al. 2020) and improves markers of hepatic function in T2DM patients (Nahra et al. 2021). The compound BI456906 has also recently entered phase II trials with a direct comparison to semaglutide (Boehringer Ingelheim 2019). Weight loss efficacy of existing GLP1R/GCGR co-agonists does not appear greater than that achieved by best-in-class GLP1R agonists, but these co-agonists may prove particularly useful in the treatment of individuals with obesity and concomitant liver disease.

2.4 GLP1R/GIPR Co-agonism

GIP was long-ignored as a therapeutic target for obesity because of studies linking loss of GIPR signalling to protection from adiposity, and a demonstrated clinical

inefficacy in patients with uncontrolled T2DM (Chia et al. 2009; Nauck et al. 1993). Although native GIP and GIPR agonists have at best moderate effects on food intake or body weight (Coskun et al. 2018; Mroz et al. 2019), dual incretin agonists which activate both the GLP1R and GIPR have recently been demonstrated to have greater efficacy in restoring normoglycaemia and reducing body weight than GLP1R agonists alone. The first unimolecular GLP1R/GIPR agonist was the pegylated DPP4-resistant compound NNC0090–2746 (Finan et al. 2013), which evoked similar improvements in HbA_{1c} and body weight to liraglutide in T2DM patients, while also reducing total cholesterol levels (Frias et al. 2017). More striking results have recently been achieved with the dual agonist tirzepatide, a once-weekly injectable peptide biased towards GIPR activation (Coskun et al. 2018). Tirzepatide reduces hyperglycaemia and body weight to a greater extent than the GLP1R agonist comparator dulaglutide, with over a third of T2DM patients achieving over 10% reduction in body weight when treated with the highest dose of tirzepatide (Frias et al. 2018). On-going phase III trials are assessing the cardiovascular outcomes of tirzepatide in T2DM (SURPASS), and the weight loss effects in non-diabetic individuals with obesity (SURMOUNT).

Given this clinical efficacy of dual GLP1R/GIPR agonists, recent work has focused on understanding the underlying mechanisms. In terms of glucose tolerance, improved glycaemic control – which could be initially mediated by GLP1R agonism – is known to re-sensitise pancreatic β-cells to the effects of GIP in T2DM patients (Hojberg et al. 2009). As GIPR antagonists have also been demonstrated to induce weight loss in some (Killion et al. 2018), but not all (Mroz et al. 2019; West et al. 2021), preclinical models, chronic activation of the GIPR has been proposed to cause receptor desensitisation which antagonises endogenous GIP activity (Killion et al. 2020); however, there is currently no substantial evidence to support this hypothesis. Chemogenetic activation of hypothalamic GIPR-expressing cells, which only show a small degree of overlap with neuronal GLP1R expression, acutely reduces food intake in mice (Adriaenssens et al. 2019). A recent publication demonstrated c-fos-staining, marking neuronal activation, in hypothalamic neurons in response to a peripherally administered GIPR agonist; this agonist reduced food intake and weight gain in DIO mice, an effect that was abolished when central GIPR expression was conditionally knocked out through a *Nestin*-Cre approach (Zhang et al. 2021). The importance of synergistic activation of GIPR- and GLP1R-expressing neurons and downstream anorexigenic pathways, and the potential of GIP for the treatment of obesity in humans, remains an area of active study.

2.5 GLP1R/GCGR/GIPR Triple Agonism

Monomeric drugs which act as triple agonists at the GIP, GLP-1 and GCG receptors have also been developed, in an attempt to combine the beneficial effects of GLP1R/GCGR and GLP1R/GIPR co-agonists. These tri-agonists induce greater body weight loss (up to 30% in DIO mice) alongside improved glucose tolerance and a reversal of steatohepatitis (Finan et al. 2015). Given its effects on liver fat and fibrosis, the

tri-agonist HM15211 has now entered phase II trials for the treatment of NASH (Hanmi 2020). Many additional compounds have also been developed and are progressing through preclinical and phase I pipelines for treatment of T2DM (Lilly 2021).

2.6 GLP1R/NPY2R Co-agonism

Several studies have demonstrated additive or synergistic effects of GLP-1 receptor agonists with $PYY_{3–36}$ (De Silva et al. 2011; Neary et al. 2005; Talsania et al. 2005). In humans, acute (150 min) intravenous infusions of native $PYY_{3–36}$ in conjunction with GLP-1 reduced energy intake (Schmidt et al. 2014). Similarly, subcutaneous infusion of GLP-1, oxyntomodulin and $PYY_{3–36}$ over 28 days in overweight individuals improved glycaemic control and caused greater weight loss than semaglutide, but it is impossible to delineate the contribution of each peptide in this study (Behary et al. 2019). High doses of native $PYY_{3–36}$ induce significant nausea, so doses of PYY analogues would have to be carefully titrated, as is currently done for GLP1R agonists (Gantz et al. 2007). Long-acting analogues of $PYY_{3–36}$ have been developed and reported to reduce food intake and body weight in primate (Rangwala et al. 2019) and rodent (Lear et al. 2020) models of obesity, alone and in conjunction with GLP1R agonists. Unlike GIP and glucagon, no unimolecular GLP1/$PYY_{3–36}$ co-agonists have been reported but co-administration of the long-acting PYY analogue PYY 1875 with semaglutide is currently being assessed in phase I human studies for obesity (Novo Nordisk 2019).

2.7 CCK1R Agonism

CCK also has potent anorexigenic effects and several CCK1R agonists were developed in the 2000s as satiety agents (Cawston and Miller 2010). While many of these drugs reduced food intake and body weight in preclinical models, they were deemed no more effective than diet alteration in human studies and were therefore discontinued (Jordan et al. 2008). Co-administration of CCK1R and GLP1R agonists (Trevaskis et al. 2015), or unimolecular co-agonists (Hornigold et al. 2018; Irwin et al. 2015), induces impressive (up to 28%) weight loss in rodent models. A new long-acting CCK1R agonist which reduces food intake and body weight in the obese mini-pig model has recently been developed (Christoffersen et al. 2020). This has not yet progressed to clinical trials but represents a promising compound for use in conjunction with GLP1R agonists if the preclinical efficacy is sustained in longer-term human studies.

2.8 Co-administration of GLP-1 and Amylin Receptor Agonists

Amylin is an anorexigenic hormone co-secreted with insulin from pancreatic β-cells, also expressed in the hypothalamus and, likely at lower levels, in EECs (Boyle et al. 2018; Habib et al. 2012; Li et al. 2015). The area postrema of the brainstem is thought to be critical for the satiety effects of amylin, although other brain regions including the hypothalamic arcuate/ventromedial nuclei and ventral tegmental area may influence control of hedonic feeding (Boyle et al. 2018). Several amylin receptors exist, which are formed when the calcitonin receptor complexes with one or more receptor activity modifying proteins (RAMPs) (Hay et al. 2018). The amylin analogue pramlintide evokes small (typically <5%) reductions in body weight in obese subjects (Aronne et al. 2007). It also induces modest improvements in glycaemic control via inhibition of gastric emptying and suppression of glucagon secretion, and has therefore been approved for treatment of both type 1 and type 2 diabetes (Ryan et al. 2005). A newer once-weekly amylin analogue cagrilintide has demonstrated efficacy in early clinical trials for obesity, alone (phase II) (Fletcher et al. 2021) or in combination with semaglutide (phase Ib) (Enebo et al. 2021). Initial results show a promising additive effect of GLP-1 and amylin receptor agonism, but this has yet to be confirmed in large-scale trials.

2.9 Summary of Gut Hormone Mimetic Treatments

Incretin mimetics have been used clinically for over a decade and are effective in improving glucose tolerance for patients with T2DM with a tolerable safety profile. The potent anorexigenic effects of the once-weekly GLP-1R agonist semaglutide and dual GLP1R/GIPR co-agonist tirzepatide in recent phase III trials represent ground-breaking opportunities for the pharmacological treatment of obesity. These drugs can induce clinically meaningful weight loss of 10–20% in overweight and obese patients, with or without diabetes, and are likely also to confer other beneficial effects such as reduction of cardiovascular mortality. Current pharmaceutical development is focused on combining GLP1R agonism with activity at other hormone receptors to further improve these results, ameliorate other complications of obesity such as NASH and minimise adverse effects.

3 Bariatric Surgery

Currently, bariatric surgical rearrangements of the gastrointestinal tract remain the most successful means of inducing weight loss in patients with severe obesity. Several mechanisms have been proposed to underlie these metabolic changes (reviewed in chapter “Bariatric Surgery”) but a key driver is accelerated nutrient delivery to the distal small intestine and subsequent dramatic increase in secretion of gut hormones such as GLP-1, PYY and oxyntomodulin (Holst et al. 2018; Larraufie et al. 2019). Postprandial excursions of GLP-1, PYY and CCK are strongly elevated

immediately post-operatively (Laferrere et al. 2007; Peterli et al. 2012), with exaggerated responses maintained for as long as 20 years after surgery (Naslund et al. 1997). Clinical studies using the GLP1R antagonist exendin-9 demonstrate a clear role for GLP-1 in mediating the beneficial effects of bariatric surgery on postprandial insulin secretion and glucose tolerance (Jorgensen et al. 2013; Larraufie et al. 2019; Salehi et al. 2014), but the importance of post-surgical PYY elevation has been studied in less detail. Following Roux-en-Y gastric bypass (RYGB), double *Glp1r/Npy2r* knockout mice achieve similar weight loss to wild-type animals (Boland et al. 2019); however, in human RYGB patients the combination of the GLP1R-antagonist exendin-9 and DPP-4 inhibition increases food intake, whilst each treatment alone is ineffective (Svane et al. 2016).

It has been hypothesised that increased circulating GLP-1 and PYY may be the result of altered exposure of the gastrointestinal epithelium to nutrients after surgery leading to changes in stimulus responsiveness or EEC differentiation. Although immunohistochemistry studies in rats (Mumphrey et al. 2013) and humans (Rhee et al. 2015) have reported small changes in L-cell number or density following RYGB, these are insufficient to explain the substantial increase in circulating levels of GLP-1 and PYY. Furthermore, in lean human and mouse models of bariatric surgery there were no major differences in EEC peptide content or transcriptome before and after gastrectomy in humans or mice (Larraufie et al. 2019), suggesting that altered nutrient flow is the critical factor underlying enhanced gut hormone secretion.

4 Stimulating Endogenous EEC Secretion

Pharmacologically stimulating endogenous secretion of anorexigenic and insulinotropic hormones or selectively expanding L-cell number could potentially mimic the positive effects of bariatric surgery. In addition to nutrient stimulation, it is possible to directly target the sensory machinery employed by electrically active enteroendocrine cells (Fig. 1). Although existing trials have focused on treating T2DM, the profound weight loss induced by RYGB and GLP-1 receptor agonism is proof of principle that the gut hormone axis is also a promising target for anti-obesity drugs.

4.1 Nutrient Encapsulation

Initial attempts to stimulate endogenous gut hormone secretion focused on oral delivery of nutritional stimuli. Oral ingestion of the amino acid glutamine modestly increases circulating GLP-1 concentrations (Greenfield et al. 2009) and improves postprandial glycaemic control in T2DM (Samocha-Bonet et al. 2011). Use of a slow-release enteric coating to reduce dosage by selectively delivering glutamine to the L-cell-rich distal gut evoked a small increase in circulating GLP-1 and insulin in

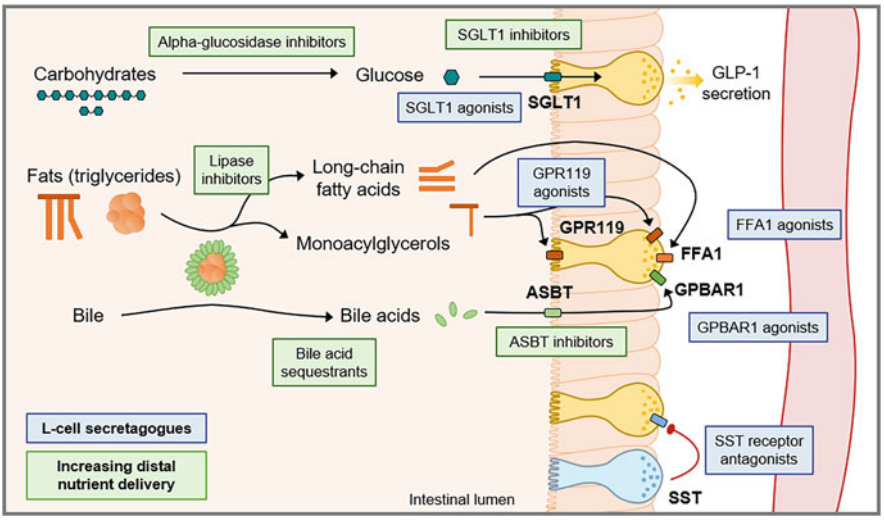

Fig. 1 Mechanisms of stimulating endogenous enteroendocrine cell secretion

fasted individuals, but no metabolic improvements were seen following an oral glucose tolerance test (Meek et al. 2016).

Similar enteric capsules have been used for ileocolonic delivery of free fatty acids and bile acids, in attempt to mimic the rerouting of nutrients observed with bariatric surgery. In fasted T2DM subjects, addition of encapsulated lauric acid to a meal lowers postprandial glucose and evokes a small increase in GLP-1 (Ma et al. 2013). Hydrolysed pine nut oil capsules slightly increase GLP-1 and GIP responses to oral glucose in healthy and overweight volunteers, compared to placebo capsules (Sorensen et al. 2021). Neither study demonstrated notable alterations in circulating insulin levels, likely reflecting inadequate stimulation of incretin release. Acute infusion of the bile salt taurocholate into the rectum potently increases GLP-1, PYY and insulin concentrations, while reducing ad libitum food intake and plasma glucose (Adrian et al. 2012). However, the high doses required for beneficial metabolic effects caused adverse reactions, including rectal irritation and abdominal pain. In a 28-day trial of participants with obesity or T2DM, ileocolonic delivery of conjugated bile acid capsules evoked small but significant improvements in glucose homeostasis, likely mediated by modest increases in GLP-1 (Calderon et al. 2020).

In developing treatments for obesity, it is important to avoid therapeutics with an inherently high caloric load, which would likely negate any beneficial anorexigenic effects. Given the limited success of using nutritional stimuli to evoke secretion of GLP-1, compared to the major elevations following bariatric surgery, efforts have largely shifted towards the use of pharmacological agonists to selectively target receptors on EECs.

4.2 Pharmacological Targeting of Enteroendocrine GPCRs

G-protein coupled receptors (GPCRs) make a significant contribution to regulation of gut hormone secretion in response to products of fat or protein digestion, neurotransmitters and other paracrine or endocrine signals. Activation of EEC receptors coupled to Gq or Gs, leading to Ca^{2+} and cyclic adenosine monophosphate (cAMP) elevation respectively, is widely recognised to stimulate hormone release. Bile acids strongly evoke gut hormone secretion via the Gs-coupled G-protein bile acid receptor GPBAR1 (Brighton et al. 2015), and monoacylglycerols also act via cAMP signalling downstream of GPR119 (Moss et al. 2016). Several Gq-coupled receptors are important for the postprandial stimulation of gut hormone secretion: the long-chain fatty acid receptors FFA1/GPR40 (Edfalk et al. 2008) and FFA4/GPR120 (Hirasawa et al. 2005), the short chain fatty acid receptor FFA2/GPR43 (Tolhurst et al. 2012), and the aromatic amino acid-responsive calcium sensing receptor CaSR (Pais et al. 2016) and GPR142 (Lin et al. 2016).

Given the inherently druggable nature of GPCRs alongside their cell surface accessibility and relatively localised expression in specific cell populations, these receptors represent a key target in the development of novel therapeutics to enhance endogenous enteroendocrine secretion. Several synthetic compounds which target EEC receptors have been developed to stimulate gut hormone release in attempts to mimic the weight loss effects of bariatric surgery.

4.3 FFA1 (GPR40) Agonists

Agonists of the free fatty acid receptor FFA1/GPR40 were initially developed for their direct effect on pancreatic β-cells (Itoh and Hinuma 2005). The partial agonist TAK-875 showed promising results in T2DM patients, increasing insulin secretion and improving glycaemic control without inducing hypoglycaemia (Burant et al. 2012); however, signs of hepatotoxicity during large-scale phase III trials halted development of TAK-875 (Kaku et al. 2016). There is currently no evidence to suggest these adverse effects were FFA1-mediated (Otieno et al. 2018) and, as liver expression of this receptor is negligible (Briscoe et al. 2003), it is hoped that newer drugs will avoid similar hepatic injury.

An example of a full FFA1 agonist is AM-1638, which evokes GIP and GLP-1 secretion, and causes GLP1R-dependent reductions in plasma glucose in mice (Luo et al. 2012). The structurally-related AM-6226 improves glucose tolerance in a primate model, more effectively than the DPP4 inhibitor sitagliptin (Brown et al. 2018). Other FFA1 agonists in preclinical development which stimulate GLP-1 secretion include SCO-267 (Ueno et al. 2019) and ZYDG2 (Jain et al. 2018). Given its dual role in insulin and incretin secretion, FFA1 presents a promising target for the treatment of T2DM. Whether newer agonists are able to stimulate sufficient release of GLP-1 and other anorexigenic hormones to evoke meaningful effects on body weight remains to be determined.

4.4 GPR119 Agonists

GPR119, the Gs-coupled monoacylglycerol receptor, is also expressed in both enteroendocrine and pancreatic α and β-cells (Chu et al. 2007; Moss et al. 2016). Preclinical studies with the agonist AR231453 demonstrate GPR119-dependent stimulation of GLP-1, GIP and PYY release, and improvements in glucose tolerance which were reduced in *Glp1r* knockout mice (Chu et al. 2008; Flock et al. 2011). Intriguingly, GPR119 agonism also slows gastric emptying independently of GLP1R, GIPR, GLP-2R and NPY2R (Flock et al. 2011). Nasogastric delivery of the endogenous GPR119 agonist 2-oleoylglycerol in humans induces a small increase in GLP-1 and GIP levels, but this was insufficient to alter insulin or glucose profiles (Hansen et al. 2011).

Despite promising early animal data, the effects of GPR119 agonists on glycaemic control in diabetic populations have been inconclusive. The agonist JNJ-38431055 stimulated GLP-1 and GIP release without altering insulin (Katz et al. 2012). Another agonist GSK1292263 increased circulating PYY approximately five-fold in T2DM subjects but did not affect GLP-1, GIP or glucose levels (Nunez et al. 2014). While this increase in anorexigenic PYY is comparable to postprandial levels after bariatric surgery, there were no differences in self-reported hunger scores. In a phase II trial of Japanese T2DM patients, the agonist DS-8500a improved Hba1c levels, to a lesser extent than sitagliptin, alongside beneficial improvements in lipid and cholesterol (Yamada et al. 2018). The same drug did not alter glycaemic control in a North American cohort when co-dosed with metformin (Sankyo 2018). There are no on-going listed clinical trials of GPR119 agonists for T2DM or obesity, but the agonist MBX-2982 recently entered phase II trials to evaluate whether it can enhance glucagon secretion during insulin-induced hypoglycaemia in type 1 diabetes (CymaBay 2020).

4.5 GPBAR1 (TGR5) Agonists

Activation of the Gs-coupled G-protein bile acid receptor GPBAR1 (previously known as TGR5/GPR131) evokes release of GLP-1, PYY, GIP, GLP-2 and insulin (Bala et al. 2014; Kuhre et al. 2018; Parker et al. 2012b; Thomas et al. 2009). Furthermore, there is preclinical evidence that GPBAR1 agonism may enhance resting brown adipose thermogenesis and decrease inflammation, providing additional benefits in the treatment of obesity (van Nierop et al. 2017).

Bile acids and selective GPBAR1 agonists stimulate GLP-1 secretion and improve glucose homeostasis in wild-type, but not *Gpbar1* knockout, mice (Thomas et al. 2009). Colorectal infusion of bile acids also increases PYY/GLP-1 secretion in humans and suppresses appetite in both T2DM and healthy-weight volunteers (Adrian et al. 2012); however, in contrast to animal studies, oral or intrajejunal dosing of bile acids in humans induces very little GLP-1 release with no effect on insulin levels (Hansen et al. 2016; Meyer-Gerspach et al. 2013). In the only published clinical study using a selective GPBAR1 agonist, T2DM subjects were

treated with SB-756050 for 6 days (Hodge et al. 2013). This trial produced disappointing effects on glucose tolerance, although there was some indication of increased GLP-1 secretion at certain doses in response to acute GPBAR1 stimulation.

GPBAR1 is widely expressed, and care must therefore be taken to balance the potential therapeutic benefits of receptor activation (improved glucose homeostasis, reduced inflammation) with the risk of diverse off-target effects. These adverse reactions have primarily been studied in animal or cell models but include gallstone formation, cardiovascular alterations, constipation, pruritus and promotion of cell proliferation [reviewed in van Nierop et al. (2017)]. It was initially hoped that it may be possible to target the enteroendocrine cell GPBAR1 directly from the gut lumen with a non-absorbable agonist, to minimise effects in other organ systems; however, it has since been demonstrated that activation of GPBAR1 occurs at the basolateral surface of L-cells, following absorption across the epithelial layer by the apical sodium-dependent bile acid transporter (ASBT) (Brighton et al. 2015).

4.6 Bile Acid Sequestrants and ASBT Inhibitors

Given the challenge of selectively targeting GPBAR1 in EECs pharmacologically, other approaches which alter the intestinal availability of endogenous bile acids may prove effective in the treatment of obesity. The majority (~95%) of bile acids are actively reabsorbed by the ASBT in the distal small intestine and returned to the liver via the portal vein for re-secretion, although a small proportion enter the systemic circulation (Dawson et al. 2009). Bile acids which reach the colon can be modified by the microbiota to form passively-absorbed unconjugated bile acids (Mekhjian et al. 1979), or lost in faeces.

Bile acid sequestrants are non-absorbable resins designed to increase faecal loss of bile acids and therefore increase de novo synthesis to reduce total plasma cholesterol. As well as improving dyslipidaemia, the sequestrant colesevelam also significantly lowers glycaemia in T2DM patients (Bays 2011), an effect which was GPBAR1-dependent in mice (Potthoff et al. 2013). Sequestrants increase delivery of bile acids to the distal intestine and slightly increase postprandial GLP-1 and GIP levels in T2DM patients (Beysen et al. 2012; Brufau et al. 2010); although this is not to the extent seen following bariatric surgery, likely reflecting the need for bile acid absorption for incretin secretion. A recent study of post-RYGB subjects also demonstrated that addition of colesevelam to a meal does not alter circulating GLP-1 levels, arguing against an important role for endogenous bile acids in mediating postprandial GLP-1 release in this cohort (Jonsson et al. 2021).

ASBT inhibitors which block small intestinal bile acid absorption have also been trialled. Although ileal GPBAR1-mediated hormone secretion depends on bile acid absorption via ASBT (Brighton et al. 2015), passive absorption of secondary bile acids in the colon – where there is a large pool of GLP-1 and PYY positive EECs – is likely to still enable GPBAR1 activation (Billing et al. 2019). In diabetic rats, oral administration of ASBT inhibitors promoted GLP-1 and insulin release (Chen et al.

2012). Patients with chronic constipation treated with high doses of the ASBT inhibitor elobixibat for 14 days showed slightly elevated postprandial GLP-1 levels (Rudling et al. 2015). The inhibitor GSK2330672 reduced fasting plasma glucose levels in T2DM subjects, although GLP-1 levels were not measured (Nunez et al. 2016). Notwithstanding gastrointestinal side effects, primarily diarrhoea, ASBT inhibitors are still under development for the treatment of hypercholesterolaemia, NASH and functional constipation. The impact of these drugs on energy intake and body weight should be monitored as they progress through clinical trials, although preclinical studies showed little alteration (Rao et al. 2016).

4.7 Slowing Macronutrient Digestion

Macronutrient breakdown is required for both absorption and stimulation of EEC secretion. Therapies which slow nutrient digestion may therefore reduce the total calories absorbed, while increasing nutrient availability in the GLP-1 and PYY-rich regions of the ileum and colon. Orlistat, a lipase inhibitor approved for the treatment of obesity, reduces breakdown of lipids to fatty acids and monoacylglycerols and delivers a large fat load distally. In healthy volunteers, orlistat taken before a meal reduces circulating GLP-1, PYY and CCK while also reducing satiety (Ellrichmann et al. 2008). The requirement for lipid digestion and subsequent absorption of fatty acids to access basolaterally-located FFA1 (Christensen et al. 2015) appears to limit the ability of orlistat to enhance gut hormone release. Alpha-glucosidase inhibitors, such as acarbose and miglitol, are approved therapies for T2DM which slow the digestion of starch and sucrose to reduce postprandial glucose elevations. These drugs appear to evoke small increases in GLP-1 after a meal or sucrose ingestion, while reducing GIP secretion from the proximal gut (Narita et al. 2012; Seifarth et al. 1998). Postprandial effects on plasma GLP-1 have not been observed in every study (Hucking et al. 2005) and, like orlistat, the efficacy of alpha-glucosidase inhibitors is likely limited by the need for carbohydrate digestion to stimulate EECs in the distal gut.

4.8 SGLT1 Inhibitors

Glucose, from ingested carbohydrates or free sugars, is absorbed across the intestinal epithelium by the apical sodium-dependent glucose cotransporter SGLT1. In electrically active EECs, this coupled uptake of glucose and positively charged sodium ions results in membrane depolarisation and subsequent hormone release (Parker et al. 2012a). Paradoxically, blocking SGLT1 leads to a pronounced increase in circulating GLP-1 at later time points, as well as reducing postprandial glucose excursions (Powell et al. 2017). Following preclinical success, the non-absorbable SGLT1 inhibitor LX-2761 has entered phase I trials as an oral anti-diabetic agent (Lexicon 2018). In parallel to ASBT inhibition, it is hypothesised that blocking proximal uptake increases the delivery of glucose to the distal intestine. As SGLT1

can no longer mediate GLP-1 release in response to glucose, alternative glucose-sensing mechanisms or increased availability of glucose-derived metabolites may be important for EEC activation in this setting.

4.9 Somatostatin Receptor Antagonists

An alternative strategy involves using somatostatin receptor (SSTR) antagonists to release tonic inhibition of enteroendocrine secretion (Jepsen et al. 2019). Specifically, a selective antagonist of SSTR5 – which is highly enriched in ileal L-cells (Moss et al. 2012) – has been shown to improve glycaemic control in mice, in a GLP-1-dependent manner (Jepsen et al. 2021; Sprecher et al. 2010). In rodents, SSTR5 antagonists only improve glucose tolerance when administered orally, further implicating an incretin-like effect rather than direct stimulation of pancreatic insulin secretion (Jepsen et al. 2021). SSTR5 expression does appear to be higher in human than rodent β-cells, and so antagonism may have a dual benefit of stimulating gut hormone and insulin release in man (Farb et al. 2017). Several SSTR antagonists have recently been developed for oral administration (Hirose et al. 2017), but clinical studies for these drugs in obesity or T2DM have not yet been reported.

4.10 Future Directions in Manipulating Endogenous EEC Secretion

The success of L-cell secretagogues in preclinical and clinical studies has been limited to relatively small increases in circulating gut hormone levels. Injectable GLP-1 analogues and bariatric surgery both induce ~ten-fold elevations in concentrations of circulating GLP-1 or equivalents, leading to potent anorexigenic effects (Calara et al. 2005; Yousseif et al. 2014). It appears clear that increasing endogenous gut hormone secretion to these levels will require synergistic activation of multiple pathways. For example, single oral administration of an SSTR5 antagonist, GPBAR1 agonist or FFA1 agonist in mice induces modest (<five-fold) stimulation of GLP-1 secretion (Briere et al. 2018). By contrast, co-treatment with a GPBAR1 or FFA1 agonist (to directly evoke GLP-1 release), a SSTR5 antagonist (to release L-cell inhibition) and a DPP4 inhibitor (to prevent breakdown) elevated circulating GLP-1 to supraphysiological levels, far greater than those achieved post-surgery or during treatment with exogenous incretin mimetics. Similarly, the use of a lipid nanocarrier system for oral exenatide delivery has recently been shown to induce enhanced GLP-1 secretion, synergistically improving glucose tolerance compared to subcutaneous exenatide (Xu et al. 2020). An alternative approach could combine basolateral activation of GPBAR1 or FFA1 from the circulation with a non-caloric drug targeting receptors on the apical processes of open-type EECs, such as the electrogenic glucose transporter SGLT1.

In recent years, bulk and single cell RNA sequencing of human EECs has been carried out using immunolabelled dissociated tissue, or genetically modified reporter organoids (Beumer et al. 2020; Goldspink et al. 2020; Roberts et al. 2019). L-cells

express several receptors not previously implicated in postprandial nutrient sensing or neurohormonal regulation, which may represent novel targets for the selective manipulation of endogenous gut hormone secretion.

5 Enhancing EEC Number

Intestinal epithelial cells – exposed to the extreme chemical and physical conditions of the intestinal lumen – have a rapid turnover time, typically only 3–5 days (Darwich et al. 2014). If it were possible to selectively enhance EEC differentiation, this could represent a promising strategy to increase endogenous gut hormone release. Several studies have characterised which transcription factors (TFs) are expressed by specific enteroendocrine cell subtypes and the time course of this expression (Beumer et al. 2020; Gehart et al. 2019). The hormonal repertoire of individual EECs is much broader than suggested by the classical single letter (e.g., 'L'-cell) classification system and expression of specific hormones is dependent on a combination of factors, including the cell's location within the gastrointestinal tract, position along the crypt-villus axis and possibly extrinsic influences such as nutritional status. A number of compounds – such as short chain fatty acids and bile acids – have been proposed to physiologically modulate EEC differentiation in organoid and mouse models (Lund et al. 2020; Petersen et al. 2014). Although attempts have been made to develop drugs which boost EEC number (Beumer et al. 2018; Petersen et al. 2015, 2018; Tsakmaki et al. 2020), significant further work is required to selectively target TFs active in specific EEC populations before this approach could be considered for therapeutically increasing gut hormone secretion.

6 Conclusion

As evidenced by recent trials of the GLP-1 receptor agonist semaglutide and the GLP-1/GIP receptor dual agonist tirzepatide, the anorexigenic and insulinotropic gut hormone axes can be effectively manipulated to induce significant weight loss in human subjects. Directly targeting the gut for the treatment of obesity also remains an attractive therapeutic strategy. Pharmacological agonists activating multiple gut hormone receptors, which have demonstrated early clinical success, could be replaced by effective stimulation of plurihormonal EECs. Increasing hormone release from the gut would also enable the activation of local receptors, such as those on vagal afferent neurons, which appear to underlie at least some of the actions of EEC products. By synergistically manipulating several pathways controlling endogenous enteroendocrine secretion, it is theoretically possible to mimic the effectiveness of bariatric surgery in a pill.

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