Chapter 6
Genetics of Coronary Artery Disease in Diabetes Mellitus

Mario Luca Morieri and Alessandro Doria

Leveraging Genetics to Decrease the Cardiovascular Burden of Diabetes

Despite the improvements in cardiovascular preventive strategies and the resulting overall decrease in cardiovascular mortality and morbidity that have occurred during the past few decades, patients with diabetes remain at higher risk of cardiovascular disease (CVD) than non-diabetic subjects [1, 2]. This, combined with the ongoing worldwide increase in diabetes prevalence, represents a global health threat with important social and financial implications. Patients with type 2 diabetes (T2D), who are 90–95% of diabetic subjects, often have other cardiovascular risk factors such as hypertension, dyslipidemia, and obesity; however, the increased CVD risk associated with T2D is independent of these other predisposing clinical characteristics, meaning that patients with T2D are at higher CVD risk than patients without diabetes even after accounting for these classic cardiovascular risk factors—an observation that is also true for hyperglycemia [2]. Understanding the mechanisms underlying this increased cardiovascular risk is of pivotal importance in order to achieve a meaningful reduction of CVD in T2D.

One approach to expand knowledge in this field is to search the human genome for variants that are associated with an increased risk of CVD in diabetes, and use the information about the location and function of these variants to infer about the mechanisms involved in the diabetes-induced acceleration of atherogenesis. This information

M. L. Morieri
Department of Medicine, University of Padova, Padova, Italy

A. Doria (✉)
Research Division, Joslin Diabetes Center, Boston, MA, USA

Department of Medicine, Harvard Medical School, Boston, MA, USA
e-mail: Alessandro.Doria@joslin.harvard.edu

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can also be used to improve the identification of T2D individuals who are at especially high risk of CVD and to personalize the preventive interventions that can be targeted at them. Although 99.5% of the 3 billion base pairs of the human genome are identical among different individuals, the 0.5% that is variable translates into millions of genetic variants potentially affecting susceptibility to common disorders. Since the complete sequencing of the human genome in the early 2000, our capability to investigate this genetic variability, and its relationship with CVD in the general population as well as in patients with diabetes, has exponentially increased. Over the past 20 years, we have moved from family-based studies, including a few hundred subjects, to large population-based studies including thousands or even millions of individuals. At the same time, we have gone from studying few variants at pre-specified loci to genotyping millions of variants covering the entire genome at progressively lower costs. Such extraordinary increase in capabilities has led to the discovery of hundreds of new genes involved in the pathophysiology of CVD, and especially of coronary artery disease (CAD). These discoveries have revealed a remarkably complex polygenic background of CAD, and although we are far from completely dissecting such complexity, we have started to leverage these findings to develop new approaches to improve prevention and treatment of CAD in patients with type 2 diabetes as discussed above.

Genetic Determinants of CAD in Diabetes

A large body of evidence points to CAD as a complex, multifactorial disease resulting from the combined effects of genetic and environmental factors. Indication of a genetic component of CAD has come from studies showing that a positive family history increases CAD risk, independently from other traditional risk factors. This has been shown to be equally the case in the general population and among patients with diabetes [3–7], with estimates of the proportion of CAD variability explained by genetic variation ranging from 40% to 60% [6, 7]. The genome-wide association studies (GWAS) that have been carried out during the past decade have shown that a large proportion of CAD heritability (40–70%) is explained by common variants (i.e., Single Nucleotide Polymorphisms—SNPs), having population frequencies greater than 5% [7–9]. However, infrequent or rare variants have also been implicated, although their effect has been more difficult to demonstrate [10–12]. The interplay between rare and common variants in shaping CAD risk is well illustrated by studies on Familial Hypercholesterolemia (FH), showing that the penetrance of the CAD phenotype of this monogenic disorder due to rare mutations is largely influenced by the polygenic component of CAD determined by common variants. For instance, in a recent study of the general population of the UK Biobank, the probability of having a CAD event by age 75 among subjects carrying rare FH pathogenic variants in the *LDLR*, *APOB*, or *PCSK9* genes varied from 17% to 78% depending on their overall genetic predisposition to CAD as estimated by a polygenic risk score combining millions of genome-wide common variants [13, 14]. Thus, from a clinical perspective, genetic susceptibility to CAD should be viewed as the combined effect of common and rare variants (Fig. 6.1). Two individuals might have a similar genetic risk of CAD, but this may derive from different combinations of rare mutations and common polymorphisms.

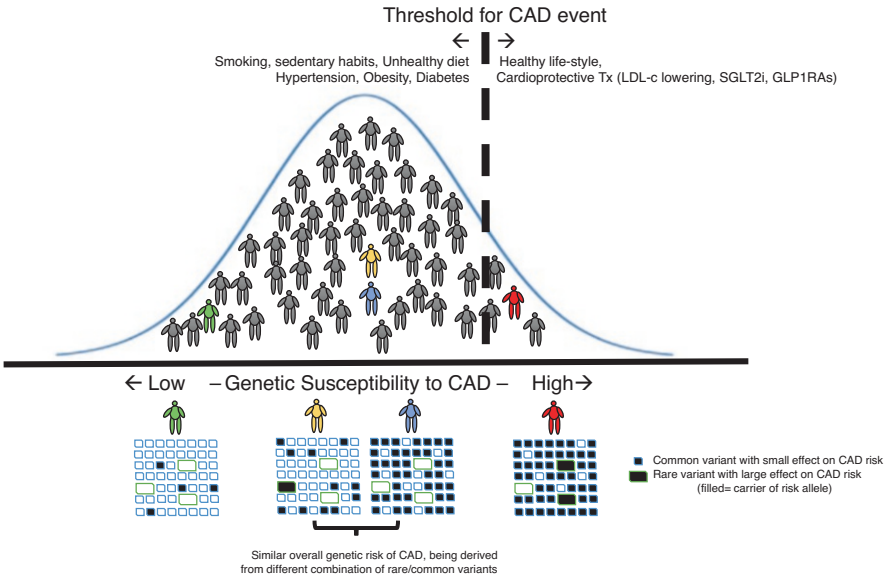

Fig. 6.1 Genetic susceptibility to CAD according to different combinations of common and rare variants at multiple genetic loci

Such genetic susceptibility to CAD acts together with the susceptibility induced by other modifiable and non-modifiable risk factors, such as male gender, smoking habits, un-healthy diet, sedentary lifestyle, presence of obesity, hypertension, and diabetes. The combined effect of these factors can move the theoretical threshold of CAD events towards the left (i.e., increasing the risk of CAD events and/or decreasing the age at which they occur), while preventive strategies (medication or health lifestyle) can move the threshold to the right (i.e., delaying or avoiding the CAD events).

As schematically illustrated in (Fig. 6.2), and discussed in more detail below, the genetic architecture of the susceptibility to coronary artery disease in patients with diabetes can be viewed as being composed by the following groups of genes: (1) Genetic variants increasing CAD risk in the general population that also increase CAD risk in subjects with diabetes. (2) Genetic variants predisposing to both T2D and CAD (in agreement with the so-called "common soil" hypothesis), and (3) Genetic variants increasing the risk of CAD specifically in the presence of T2D or hyperglycemia (i.e., through gene-by-environment interactions).

Genetic Variants Increasing CAD Risk in the General Population and in T2D

To date, common genetic variants at more than 160 loci have been found to be independently and consistently associated with CAD in GWAS that were mainly conducted in the general population and in subjects of European or Asian ancestries [15]. Whether these loci are associated with increased CAD risk also in patients

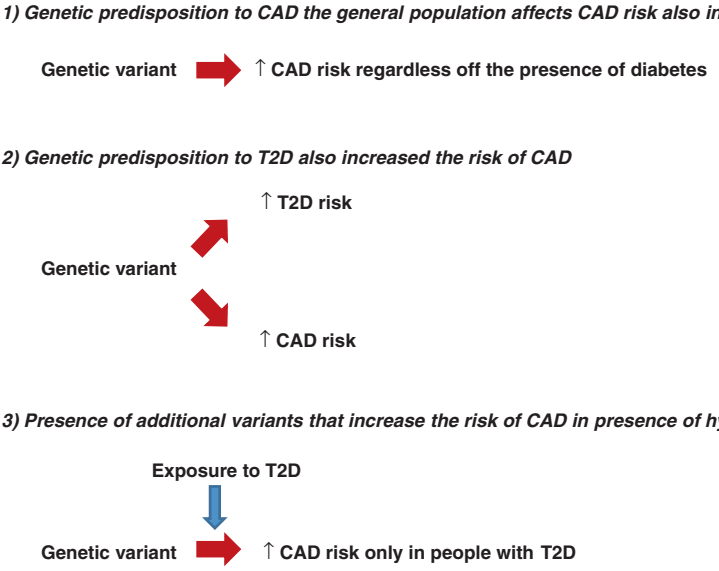

Fig. 6.2 Genetic architecture of increased susceptibility to coronary artery disease in diabetes

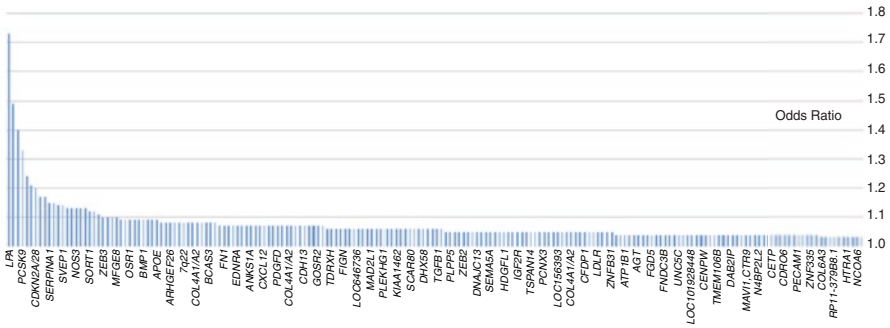

Fig. 6.3 Graphical representation of 160 CAD risk loci discovered in the general population. Loci are sorted by the strengths of their association with CAD (expressed as odds ratio per risk-allele copy). For graphical purposes, only some representative genes/loci are indicated with their names

with type 2 diabetes has been the topic of several studies [16–19]. Given the relatively small effect of each of these variants (most of them increase the risk of CAD by 5–10% per each risk allele variants, and very few increase the risk above 20%, as shown in Fig. 6.3) their validation in subjects with diabetes requires large sample sizes and well-defined phenotypes (e.g., diabetes diagnosis predating the CAD event). For this reason, the analysis of single variants has been often replaced by the analysis of genetic risk scores (GRS) capturing the overall polygenic burden of each individual. GRS can be estimated as "crude GRS," i.e., a score equal to the sum of the number of risk allele for each polymorphism, or as "weighted GRS," where each risk allele is weighted by its strength of association with CAD.

The first of these studies, conducted a decade ago, was a case-control study combining three different populations of subjects with T2D and CAD from the Joslin Heart Study, the Nurses' Health Study, and the Health Professional Follow-up Study [16]. In a combined analysis of these three cohorts, 5 of the 12 CAD loci that had been identified in the general population at that time (2011) were found to be nominally associated with CAD also among patients with T2D. A GRS derived from these SNPs showed a significant association with CAD, with a 19% increase in risk (95% CI 13–26%) for each additional risk allele in the GRS. Similar results were reported in 2015 in a post hoc analysis of the Look-AHEAD study (Action for Health in Diabetes) [17], including 4016 overweight or obese subjects with T2D who were followed for a median of 9.6 years. In this cohort, each standard deviation of a GRS derived from 153 SNPs associated with CAD in the general population was associated with a 19% increase in risk of incident CVD (95% CI 10–28%, $p = 1 \times 10^{-5}$). This GRS was also associated with classic CVD risk factors such as higher LDL-cholesterol, blood pressure, and HbA1c, but the association with CVD remained significant after adjustment for these factors.

More recently, our group has tested the association of major coronary events (combining fatal CAD events, non-fatal myocardial infarction, and unstable angina) with a GRS composed of 204 SNPs representative of the 160 CAD loci known as of 2018 to be associated with CAD in the general population at genome-wide significance level ($p < 5 \times 10^{-8}$) [19]. This analysis was conducted in white participants from the "Action to Control Cardiovascular Risk in Diabetes" (ACCORD, $n = 5360$) and "Outcome Reduction with Initial Glargine Intervention" (ORIGIN, $n = 1931$) studies. In the ACCORD study, 32 SNPs were found to be nominally associated with CAD, although they did not reach study-wide significance levels because of the limited power to analyze individual loci. In addition, 151 out of the 204 SNPs that were tested (i.e., 74% of them, corresponding to a p value 2×10^{-12} for deviation from the null hypothesis of 50%) showed the same trend of association with prevalent CAD as that reported in the general population. A weighted GRS derived from the 204 SNPs was associated with 27% ($p = 4 \times 10^{-10}$) and 35% ($p = 2 \times 10^{-4}$) higher risks of incident major CAD events per standard deviation in the ACCORD and ORIGIN studies, respectively. This GRS was also associated with family history of CVD, prevalent CVD, and HDL-cholesterol at baseline, but its association with incident CAD persisted, although slightly attenuated, after adjustment for these and other classic CVD risk factors (including the ACC/AHA Pooled Cohort Equations 10-year CVD risk model). Moreover, the association was similar in subjects in primary or secondary CVD prevention and with or without a family history of CVD.

Altogether these studies indicate that the genetic factors predisposing to CAD in the general population do so also in people with T2D, suggesting that the presence of a powerful CVD risk factor such as diabetes does not override these genetic effects. In fact, exposure to the diabetic milieu may enhance the effect of some of these genes, as exemplified by the finding of synergism between poor glycemic control and the CAD locus on 9p21 [20]. As discussed in the following section, diabetes may also provide an additional genetic burden since some of the genetic variants that predispose to T2D, and are, therefore, over-represented among diabetic subjects, may also predispose to CAD.

Genetic Variants Predisposing to Both T2D and CAD

Several studies have suggested the existence of a common genetic background ("common soil") shared by T2D and CAD [21]. Two such studies evaluated the results from separate GWAS on CAD and T2D and, after accounting for sample overlaps between studies and the linkage disequilibrium between variants, found a significant positive correlation between allelic effects on T2D and CAD risk across the genome (r_g = 0.39–0.40) [22, 23]. Of note, in one of these studies, the correlation between CAD and T2D (r_g = 0.39) was higher than that between CAD and other traits such as LDL-c, HDL-c, triglycerides, and BMI [23]. Another approach to assess the shared genetic background of T2D and CAD has been to test whether variants identified as being strongly associated with T2D are enriched with variants that are also associated with CAD. Following this strategy, Jansen et al. analyzed 22,233 CAD cases and 64,762 controls from the CARDIOGRAM genome-wide dataset (derived from the combination of several worldwide studies and consortia) and found that 10 of 44 variants (23%) known in 2015 to be associated with T2D at genome-wide significant level were nominally associated with an increased risk of CAD. This proportion was much higher than that expected by chance under the null hypothesis of no association (23% vs. 5%, $p = 5 \times 10^{-5}$) [24]. In the same study, the number of SNPs with an effect that was consistent between T2D and CAD risk (odds ratio per risk allele >1 for both conditions) was significantly higher than that expected by chance (i.e., 64% [29/44] vs. 50%, $p = 0.02$). Of note, the enrichment of T2D SNPs with variants associated with CAD was unaffected by the exclusion of those SNPs with profound effects on other known CAD risk factors. Similar conclusions were drawn by another paper conducted on an overlapping, but larger, population and with slightly higher number of SNPs [25]. A more recent study (including 106 variants associated with T2D as of 2017) confirmed the enrichment of T2D variants (31/106, 29%) with SNPs significantly and concordantly associated also with CAD risk (binomial test for chance observation $p < 5 \times 10^{-15}$) [26]. Other studies have confirmed the association between genetic predisposition to T2D and increased CAD risk in East Asian populations [27, 28].

The "Common Soil" Hypothesis and Insulin Resistance

In the studies above, the enrichment of T2D SNPs with CAD variants was similar for subset of genes affecting different T2D pathways (e.g., reduced beta cell function, reduced insulin sensitivity, or altered insulin secretion). However, more recent studies (based on the increasing number of genetic variants found to be associated with T2D) have suggested that CAD risk may not be equally increased by the different genetic mechanisms determining increased T2D risk [26, 29]. Specifically, genetic variants that increase T2D through pathways related to insulin resistance appear to be among those that are most strongly associated with CAD [30]. From the moment when the "common soil" hypothesis was put forward, insulin

resistance has been proposed as the most important link between T2D and CAD [21]. Compelling evidence for this concept has been provided by GWAS studies, showing that the genetic locus of *IRS1* (encoding insulin receptor substrate-1) harbors multiple variants increasing the risk of both CAD and T2D [22, 24, 26, 30–34]. Despite been placed more than 500,000 base pairs from the *IRS1* coding region, these variants are located in an enhancer site with long-range effects on *IRS1* expression [35] and have been associated with *IRS1* expression in human adipose tissue [33, 34] as well as with several traits related to insulin resistance, such as fasting insulin, HDL-cholesterol, triglycerides, and adiponectin levels, as well as body fat distribution [34, 36, 37]. Altogether, these data provide strong evidence of a causal role of insulin resistance not only in T2D but also in CAD. This concept is also supported by the recent discovery of novel CAD loci (e.g., rs11057401 p.Ser70Cys in *CCDC92*) associated with insulin resistance-related phenotypes such as body fat percentage, HDL, triglycerides, and adiponectin levels [33, 38].

Genetic Variants Increasing the Risk of CAD Specifically in the Presence of T2D

Beyond the genetic factors cited in the section on "Genetic Variants Increasing CAD Risk in the General Population and in T2D", some additional factors has been found to increase CAD risk specifically in the presence of diabetes or diabetes-related metabolic traits (e.g., hyperglycemia or insulin resistance). These variants therefore show a significant "gene by environment" interaction, by which the association with CAD is stronger, or exclusively present, among patients with diabetes as compared to the general population.

The 1q25 Locus

As of today, the strongest and most replicated signal showing a significant "gene by diabetes" interaction is represented by a genetic variant at the 1q25 locus associated with the expression of *GLUL* (coding for glutamate-ammonia ligase, a.k.a. glutamine synthase, converting glutamic acid to glutamine) [39–41]. This locus was initially identified by our group in a genome-wide association study specifically aimed at identifying genetic variants associated with increased CHD risk (defined as fatal or non-fatal myocardial infarction, revascularization procedures, or angiography evidence of significant coronary stenosis) in patients with type 2 diabetes [39]. The study combined a total of 1517 CHD cases and 2671 CHD-negative controls with type 2 diabetes derived from five independent population (the Nurses' Health Study—NHS, the Health Professionals Follow-up Study—HPFS, the Joslin Heart Study—JHS, the Gargano Heart Study, and the Catanzaro Study). After testing 2.5 million common variants, a genome-wide significant association was identified

between SNP rs10911021 (on chromosome 1q25) and CHD risk. The association, observed consistently across the five datasets, was such that each copy of the risk allele "C" was associated with a 36% higher risk of CHD (OR 1.36, 95% CI 1.22–1.51, $p = 2 \times 10^{-8}$) in patient with diabetes, but not among patients without diabetes (OR 0.99, 95% CI 0.87–1.13), yielding a significant "SNP by diabetes" interaction ($p = 2 \times 10^{-4}$). A similar association, although not reaching statistical significance, was found in a study by the *University College, London School of Hygiene and Tropical Medicine, Edinburgh and Bristol* (UCLEB) Consortium (including 12 prospective studies in patients mainly of European ancestries), in which the C-allele showed a non-significant trend for increasing CHD risk in patients with diabetes and without previous history of CAD (HR 1.25; 95% CI 0.94–1.66) [42]. As reported in Fig. 6.4, the meta-analyses of these results with those from our studies yielded an increase in the significance of the association with CHD (HR 1.35; 95% CI 1.24–1.47, $p = 8 \times 10^{-10}$). Also in this study, rs10911021 was not associated with CHD in patients without diabetes [42]. The association with increased CHD was confirmed also among 3295 patients with diabetes on primary CVD prevention enrolled in the prospective Look-AHEAD study [40]. In that study, conducted over 9.7 years of follow-up, the "C" risk allele was significantly associated with a 17% increased risk of CVD (HR 1.17, 95% CI 1.01–1.36, with CVD defined as a composite outcome of death from cardiovascular causes, non-fatal

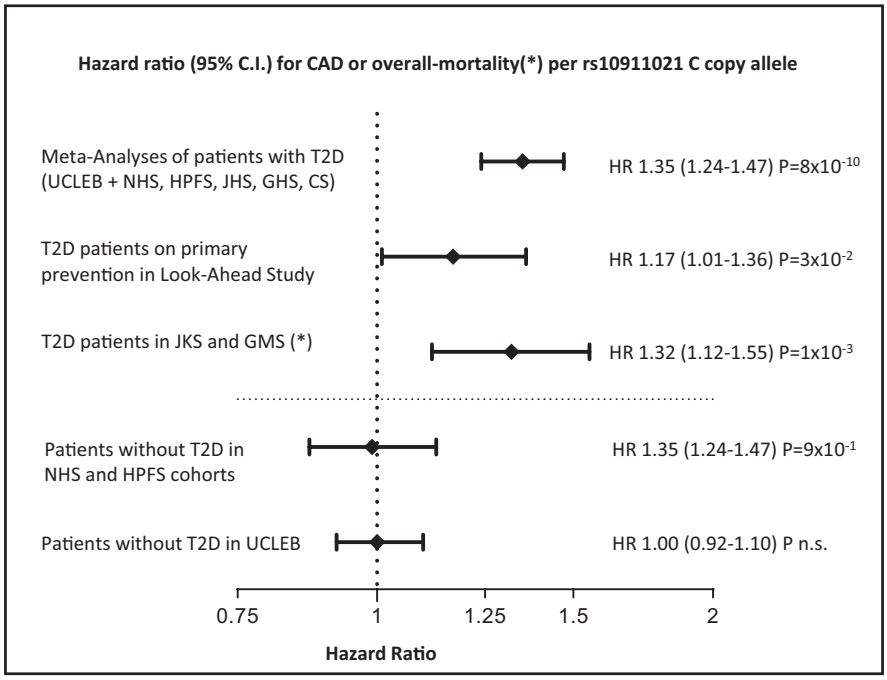

Fig. 6.4 Association between variant rs10911021 at the *GLUL* locus and clinical outcomes in longitudinal studies of patients with and without diabetes

myocardial infarction, non-fatal stroke, or hospitalization for angina). Another study, combining 1242 white subjects with type 2 diabetes from the Joslin Kidney Study (JKS) and the Gargano Mortality Study (GMS), confirmed the association of the C-allele with a higher risk of all-cause mortality (HR 1.32; 1.12–1.55, $p = 0.0011$) that appeared to be driven by cardiovascular mortality [41].

It is worth mentioning that two studies failed instead to detect a significant interaction between rs10911021 and diabetes on CAD risk [43, 44]. The design of these studies, however, was strongly biased towards the null hypothesis (i.e., finding no differences in the effect of gene on CAD between T2D and non-T2D, when instead a true difference is present) since prevalent cases of CAD were defined as having occurred among patients with diabetes regardless of whether the CAD events had occurred before or after the diagnosis of diabetes. Instead, a proper test of "gene × environment" interaction requires that the environmental factor (in this case, diabetes) is present before the onset of the outcome (in this case, CAD), and that this exposure is long enough to influence the genetic effect (meaning that duration of diabetes should be taken into account). Therefore, by including only prevalent data, and considering CAD events prior to diabetes equivalent to those after diabetes, these studies introduced a crucial misclassification that biased results towards the null hypothesis.

More recently, in support of the generally consistent and well-replicated findings of the *GLUL* locus, functional studies have increased our understanding of the mechanism of this genetic effect, suggesting a dysfunction of the γ-glutamyl cycle, leading to intracellular alteration of glutathione levels that increases susceptibility to oxidative stress. These findings point to a new potential pharmacological target to reduce CAD disease in diabetes, as described in more detail in the section on "Development of New Preventive Interventions" [45].

The *HP* Locus

Another genetic variant with a potential effect on CAD only in the presence of diabetic milieu has been identified in the *HP* gene (coding for Haptoglobin—a plasma protein binding-free hemoglobin, which, in physiological conditions, reduces hemoglobin-induced oxidative damage). This variant is a common biallelic Copy Number Variant (CNV—rs72294371), determining Haptoglobin proteins with distinct forms and lengths, leading, therefore, to three different genotypes (HP 1/1, HP 1/2, and HP 2/2) [46–48]. The HP 2/2 genotype was found to increase the risk of incident CAD risk only in patients with HbA1c above 6.5% [49]. A similar trend was reported in other prospective studies [50], and HP 2\2 was found to increase CAD risk also in patients with type 1 diabetes [51]. However, in these studies, the interaction with Hba1c levels was not formally tested, or if it was, it was not significant [50]. The evidence has been inconclusive also for the interaction between HP 2/2 genotype and intensive glycemic control on incident of CAD events among patients with type 2 and type 1 diabetes [52–54]. Therefore, further validation and replication studies are required. Nonetheless, given the role of Hp in regulation of

hemoglobin-induced oxidative damage, these findings may be consistent with those on *GLUL*, as they also seem to suggest that anti-oxidant homeostasis plays a pivotal role in modulating the onset of coronary artery disease in patients with diabetes [50].

Translating Genetic Findings to Clinical Practice

The discovery of genetic variants associated with CAD provides several opportunities to translate these findings into actionable items to improve the care of patients with diabetes. A straightforward application is the use of these genetic markers to improve prediction of CVD risk. From this standpoint, genetic markers can be considered and tested for validity, as it is done with other novel biomarkers, with the major advantages of (1) being stable over time (one test in a lifetime is sufficient) and (2) not being susceptible to reverse causation as no disease, treatment, or other modifiable factor can influence the presence of one or the other allele since these are inherited at conception. Another application relates to the discovery of new genes or pathways involved in CAD risk, which may point to new targets for cardiovascular prevention. Despite the challenges of moving from genetic associations to the identification of causal variants, causal genes, and the design of new drugs, this process has already being successful in some cases. Finally, pharmacogenetics is one of the most exciting and challenging field of investigation, holding the promise to use genetics variants to identify those subjects who might benefit the most from a specific treatment and distinguish them from those patients who would not derive benefit or may be even harmed by it. The following Sections provide some examples in each of these fields of translational research.

Improving CV Risk Assessment

Using genetic findings to improve risk prediction is an obvious application of genetic research on CVD/CAD. While individual SNPs cannot be used for this purpose due to their small effects, the GRS combining multiple SNPs that were described above may provide this opportunity. Indeed, this has been showed to be the case in a recent study of the ACCORD cohort [19], in which the GRS combining 160 CAD loci significantly improved the prediction of future CAD events when added to conventional risk factors. Although the C-statistics only minimally increased (+1%), there was a significant improvement in the correct classification of subjects in those who did and those who did not develop events during the follow-up, as shown by the substantial increase in relative Integrated Discrimination Index (rIDI + 8%, $p = 7 \times 10^{-4}$) and in the Net Reclassification Index (NRI = 0.16, $p < 1 \times 10^{-4}$). From a clinical perspective, the AHA and ACC committee used an rIDI threshold of at least 6% to evaluate whether it was useful or not to add a new biomarker to the Pooled Cohort Equations CVD risk model [55]. Therefore, this

GRS based on 204 variants (160 loci) would have passed that threshold and would have been considered for inclusion in the CVD risk equation.

An important determinant of the predictive performance of GRS's is the number of loci that are included in the scores. This is illustrated well by a retrospective analysis of the performance of the GRSs that could have been built at different times during the past decade based on the CAD-associated SNPs that were known at each point in time. As can be seen in Fig. 6.5, the increase in the number of known CAD loci that could be included in the GRS [56] has been paralleled by an increase in the prediction and discrimination provided by this tool [19]. However, since the new CAD loci that are discovered have increasingly smaller effect on CAD risk, an increasingly larger number of SNPs is required to further increase the GRS performance. This has led, over the past year, to the idea of building genome-wide polygenic risk score (GPRS) for CAD based on up to six million common variants, i.e., a GRS including all available common variants regardless of the *p* value for their association with CAD [57]. In the general population, these GPRS show better performance in discriminating subjects at very high CAD risk as compared to "classic" GRS using only genome-wide significant variants. For instance, a GPRS based on six million variants could identify a significant (8%) proportion of the population having a cardiovascular risk equivalent to that of subjects with a rare monogenic form of CAD such as familial hypercholesterolemia [57, 58]. Several studies have then confirmed the usefulness of these GPRS on top of classic risk factors for the prediction of incident cardiovascular events [59–62]. However, some other studies have yielded negative or mixed results [63, 64], highlighting the need for further research with larger sample size and multiancestry representation [12]. Also, few studies have evaluated the role of these large GPRS in patients with type 2 diabetes. In a subset of 21,102 subjects from the UK Biobank, each S.D. of a GPRS based on more than six million common variants was associated with a 50% higher risk of prevalent CAD risk (OR per SD 1.50, 95% CI 1.43–1.57), and among 352 subjects with type 2 diabetes from the McGill Cardiac Complications in Diabetes cohort (MCCD) who underwent coronary angiography, it was associated with a 65%

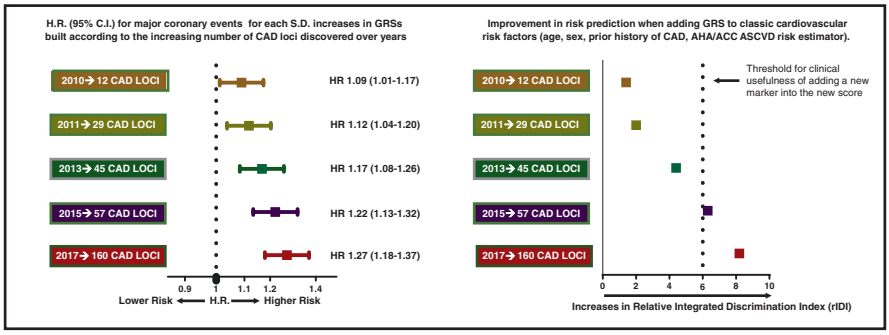

Fig. 6.5 Progressive improvement of genetic risk scores (GRS) for prediction of incident CAD events in patients with type 2 diabetes from ACCORD. Note: AHA/ACC ASCVD: 10-year American College of Cardiology (ACC)/American Heart Association (AHA) atherosclerotic cardiovascular disease (ASCVD) risk estimator. (Adapted from Morieri et al. Diabetes Care 2018 [19])

higher risk of multivessel stenosis (95% CI: 1.25–2.20) and larger number of major stenotic lesions (OR = 1.35; 95% CI 1.08–1.69) [65]. However, the performance of this GPRS in terms of improving risk prediction and discrimination over that provided by traditional risk factor has not been determined.

Overall, while more research is needed and further improvements can be expected, we can conclude that the genetic prediction tools that are available at this time, such as the GRS based on 204 SNPs [19], have reached the threshold for being adopted in clinical practice and efforts should be made to facilitate this process by incorporating them in the current clinical management guidelines.

Development of New Preventive Interventions

Several of these CAD-associated variants affect genes with well-known links to classic factors (e.g., LDL-cholesterol or blood pressure) that are known to increase CAD risk both in patients with and without diabetes [66]. However, the majority of these genetic variants are located in proximity of genes with as of yet undefined function [12]. This provides many opportunities for novel discoveries on the mechanisms regulating atherogenesis and the development of CAD in T2D, which in turn may potentially uncover novel targets for preventive strategies. At the same time, converting genetic associations into causal genes and atherogenetic pathways, and finding old or new drugs to target these, present several challenges that may delay the translation of genetic findings into clinical practice. These challenges are related to different factors, which are discussed below by describing three different genetic findings that are at different stages of translation into clinical practice: one still very far from this goal (locus 9p21), one half-way through achieving this goal (locus 1q25, *GLUL*), and one for which this goal has been achieved (PCSK9 inhibitors).

9p21 Locus

One of the clearest examples of how hard it can be to translate genetic associations into new preventive interventions is the 9p21.3 locus. This genomic region hosts the first genetic variants that were discovered to be associated with CAD by a genome-wide study back in 2007 [67, 68]. It is one of the most replicated genetic associations with CAD, including in subjects with diabetes [20, 22]. A recent meta-analysis of multiple genome-wide dataset of CAD, reported a summary Odds Ratio of 1.21 (OR = 1.21, 95% CI 1.19–1.22, $p = 5 \times 10^{-204}$) for the leading variants in this locus (rs4977574), meaning that the odds of CAD are increased by ~21% for each risk allele carried by an individual [38]. Such increased risk is unaffected by adjustment for other cardiovascular risk factors, implying that this effect is independent from known risk pathways [69]. Yet, despite these consistent and replicated findings, and despite multiple functional studies [70–75], the mechanisms linking these variants to CAD are still unclear, making the translation of this finding into an actionable target far from being achieved [12]. One of the reasons for these disappointing

results relate to the large size of the locus harboring these CAD-associated variants—a 60 kb linkage disequilibrium block with no protein-coding genes. Current evidence supports the involvement of the long non-coding RNA *CDKN2B-AS1* (a.k.a. ANRIL, for antisense non-coding RNA in the INK4 locus), located in this region and expressed in many cell types relevant to the atherosclerotic process [70, 71]. The CAD-associated variants have been found to influence ANRIL expression and splicing, increasing the supposedly pro-atherogenic, short linear ANRIL isoform and decreasing the long-circular anti-atherogenic isoform [72]. The closest protein-coding genes, which are placed outside the 60 kb locus where the CAD-associated variants are located, but could be influenced by them, code for cyclin-dependent kinase inhibitor 2A and 2B (*CDKN2A* and *CDKN2B*). The products of these genes control cell proliferation, cell aging, and apoptosis, are expressed at high levels in endothelial and inflammatory cells and may be therefore also involved in the genetic association [73–75]. *CDKN2A* and *CDKN2B* are also expressed in pancreatic islets where they play a role in regulating islet cells regenerative capacity [75], consistent with in vitro experiments showing that reduced expression of *CDKN2A* significantly modifies insulin secretion [76]. Indeed, the same locus also harbors at least two distinct genetic signals of association with a higher risk of diabetes, one of which is correlated with the CAD-associated variants [70, 77, 78]. Another element of complexity is that the 9p21 locus has been reported to have a larger effect on CAD risk among individuals with type 2 diabetes than in the general population, and among those with diabetes, to have a larger effect on among those with the worst glycemic control (as shown in Fig. 6.6) [20]. Such synergism between diabetes and the 9p21.3 locus on CVD suggests at least a partial overlap between

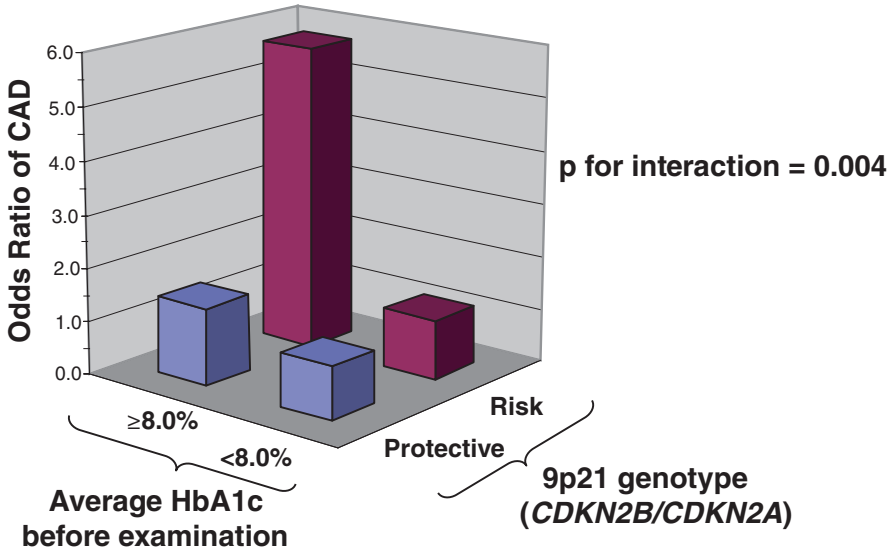

Fig. 6.6 Interaction between 9p21 CAD locus and poor glycemic control on risk of CAD in the Joslin Heart Study

the pathways through which this locus and diabetes increase CAD risk. This interaction, however, has not been replicated in other studies, some of these reporting inconclusive results [70, 79–81], and other reporting significant interactions, but in the opposite direction for glycemic control [82] and the presence of diabetes [22]. Altogether, the lack of identification of the causal variant(s), causal gene(s), and tissue(s) that are involved in the association between the 9p21.3 locus and CAD clearly illustrate the challenges of translating genetic findings into clinically actionable items. Nonetheless, the overlap between variants influencing CAD and T2D, the possible gene by diabetes and gene by glycemic control interactions, and the fact that this is the strongest CAD loci identified to data in general population, clearly warrant further studies of this locus.

1q25 Locus

In contrast with the 9p21 locus, the 1q25 locus associated with CAD in patients with diabetes provides an example of a genetic finding with a more promising path towards its possible translation into novel treatments for cardiovascular prevention. Already in the first report of the association between this locus and CAD risk in patients with diabetes, the rs10911021 C-risk allele was shown to be associated with lower endothelial expression of the nearby gene *GLUL* [39] coding for glutamine synthase—the catalytic enzyme-converting glutamate to the amino acid glutamine [83]. Although the SNP was not associated with neither glutamate nor glutamine levels, it was found to be associated with lower pyroglutamic/glutamic ratio. These two metabolites are intermediates in the γ-glutamyl cycle, which is responsible for the production and homeostasis of the anti-oxidant glutathione (GSH). On this basis, the genetic variants affecting CHD risk in patients with diabetes have been postulated to acts through this pathway [39]. This hypothesis has been further investigated and confirmed in a recent study of a large collection of human umbilical vein endothelial cells (HUVECs) naturally carrying different genotypes of rs10911021 and exposed to different glucose concentrations [45]. This study confirmed the association between the SNP and *GLUL* expression as well as its effect on a variety of metabolites related to glutamic acid metabolism and the γ-glutamyl cycle. In particular, the C-risk allele was associated with reduced GSH/glutamate ratio and was found to be inversely related to S-lactoyl-glutathione, which originates from the GSH-mediated detoxification of methylglyoxal—a glycolysis byproduct and precursor of AGEs implicated in the pathogenesis of CVD in diabetes [84]. This raised the hypothesis that the detoxification of methylglyoxal is impaired among carriers of the rs10911021 C-allele risk and is responsible for the increase in CVD risk observed among these subjects. In support of this hypothesis, the study found (1) an increase of methylglyoxal levels per each C-allele copy in HUVEC cells and (2) a significant increase in methylglyoxal levels following *GLUL* down-regulation through shRNA interference. As summarized in Fig. 6.7, these findings support the following chain of events: C-risk allele → lower *GLUL* expression → impaired γ-glutamyl cycle and glyoxalase system → and higher,

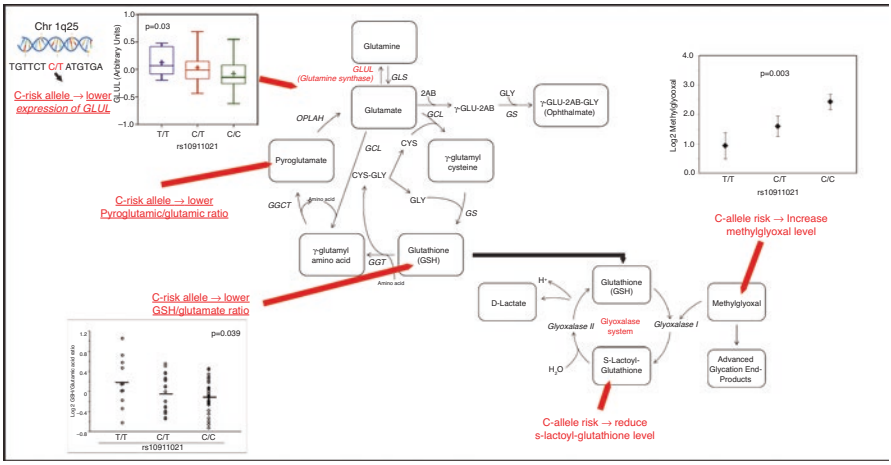

Fig. 6.7 Graphical representation of the effect of 1q25 locus variant on γ-glutamyl cycle and glyoxalase system. Amino acid levels and ratios influenced by the rs10911021 C-risk allele are indicated in red. (Adapted from Pipino et al. Diabetes 2020 [45])

pro-atherogenic, methylglyoxal levels [39]. Most importantly, from a translational point of view, the study showed that the increase in methylglyoxal levels induced by GLUL deficiency was completely prevented by exposing cells to high concentration of glutamine (the product of the GLUL regulated enzymatic reaction). Notably, oral supplementation with L-glutamine (a precursor of NAD) is an already FDA-approved treatment for sickle cell disease, in which this treatment was found to raise the NAD redox ratio in red blood cells and reduce oxidative stress [85–87]. Therefore, while further functional studies are required, these results may support the use of glutamine supplementation for cardiovascular prevention in patients with type 2 diabetes carrying the 1q25 risk allele—an approach whose usefulness can be easily investigated through a clinical trial.

PCSK9

The *PCSK9* locus is an example of a successful and completed translation of genetic finding into clinical activity, which, in less than 15 years from its discovery, has led to the development and clinical use of a highly effective new cardiovascular prevention treatment, i.e., proprotein convertase subtilisin–kexin type 9 inhibitors. The role of the *PCSK9* gene in autosomal-dominant hypercholesterolemia was initially discovered in 2003 [88] and subsequent studies quickly led to the identification of the protein coded by this gene as a regulator of LDL-receptor (LDL-r) degradation. By binding to the LDL-r, PCSK9 promotes degradation of this molecule in hepatocytes, decreasing the clearance of LDL from circulation and therefore increase circulating LDL-cholesterol levels. In only a few years, the identification of rare loss-of-function PCSK9 mutations associated with reduced

LDL-cholesterol levels and CAD risk [89, 90], in particular in African Americans, led to the development of two monoclonal antibodies targeting the PCSK9 protein and reducing LDL-cholesterol levels [91–93]. Approved for treatment of hypercholesterolemia in 2015, these inhibitors are very effective in reducing the incidence of cardiovascular events and mortality in subjects at high cardiovascular risk [94–96]. The quick translation of the genetic finding with PCSK9 into new treatments has been due to the initial link of this gene to a well-known CV risk factor (i.e., the LDL-cholesterol). It is reasonable to expect that developments will be much slower for those genetic findings for which the causal genes and pathways are not so clear. Nonetheless, the PCSK9 inhibitors story is encouraging and also provides additional hints concerning the usefulness of genetic studies. For instance, the findings of subjects carrying two loss-of-function PCSK9 alleles (i.e., with no or much reduced PCSK9 function) having no adverse health consequences supported the safety of PCSK9 pharmacological inhibition before the development of specific inhibitors and before clinical trials [97, 98]. One of the factors currently limiting the use of this cardiovascular preventive treatment is its high cost. One way to overcome this problem is to improve selection of subjects who will benefit the most from this treatment in order to prioritize treatment. In this regards, two distinct post hoc studies of randomized clinical trials have recently shown that subjects with a higher genetic risk of CAD, as identified with the use of a polygenic risk score for CAD similar to those described in the previous section, experience a higher relative and absolute risk reduction when treated with PCSK9 inhibitors [99, 100]. These early data provide the rationale for a successful pharmacogenomics approach as described in detail for other interventions in the next section.

Personalization of Therapy

Over the past few years, several studies have tried to identify new approaches to personalize cardiovascular prevention treatment in patients with type 2 diabetes. One of these approaches has been based on the hypothesis that the response to cardiovascular preventive treatment is partially determined by the genetic background of each patient. For this reason, there has been an increased interest, as part of the wider field of precision medicine, in pharmacogenetic studies aimed at identifying genetic variants associated with better or worse response to treatments. One may search for these variants through a genome-wide unbiased approach (i.e., without a priori hypotheses or set of genes being specified) or following a candidate-gene strategy (i.e., studying a set of pre-specified gene(s) or variant(s)). These approaches are complementary, since each of them has advantages and limitations. Below, we discuss two examples, one for each approach, showing promising results for implementation in clinical practice.

Genetics Determinants of Cardiovascular Response to Intensive Glycemic Control

Epidemiological studies have clearly shown the relationship between worst glycemic control and increased incidence of macro- and micro-cardiovascular disease in patients with diabetes [101]. These observational findings were confirmed in randomized controlled trials, in which interventions aimed at achieving intensive glycemic control clearly showed a benefit in reducing the incidence of micro-vascular complications [101, 102]. Results were less clear for macro-vascular disease. While meta-analyses showed that intensive glycemic control was associated with a 15% reduction in the risk of myocardial infarction and 9% reduction in major cardiovascular events [103], the effects on total and cardiovascular mortality were found to be neutral and in some cases even detrimental [101, 104]. For instance, the ACCORD clinical trial, which enrolled over 10,000 subjects, showed that participants randomized to intensive (Hba1c < 6.0%) rather than standard glycemic control (Hba1c between 7% and 8%, in line with recommendation at the time the study was conducted) experienced a significant reduction in myocardial infarction risk (−18%). However, this benefit was completely offset by a significant and paradoxical increase in total (+22%) as well as cardiovascular (+35%) mortality associated with intensive glycemic control, which led to an early termination of the trial [104]. Following these results, the achievement of intensive glycemic control with Hba1c < 6.0% has not been recommended by guidelines. In addition, the more recently discovery and approval of innovative cardioprotective glucose-lowering treatments, such as SGLT2 inhibitors and GLP-1Receptor Agonists, have not changed this recommendation. Indeed, although these drugs have shown a consistent cardiovascular benefit (including on mortality) as compared to placebo [66, 105], such an effect appears to be only in minor part related to HbA1c reduction [106–109], and these drugs are currently recommended for cardiovascular prevention according to the cardiovascular risk of patients and not to achieve lower HbA1c targets [66, 105]. Therefore, the question as to which patients might experience benefit or harm from intensive reduction of HbA1c, e.g., below 6.0%, is still unanswered.

Through genetic studies, we were able to provide an initial and promising answer to this question. Specifically, through a genome-wide analysis of over seven million common variants in self-reported white participants randomized to intensive glycemic control in the ACCORD clinical trial, we have identified two distinct genetic signals that were associated with higher risk of cardiovascular mortality at genome-wide significance levels [110]. The first of these loci is located in an intron of the *MGMT* (O-6-methylguanine-DNA methyltransferase) gene on chromosome 10 while the second is located upstream and proximal to three long intergenic non-coding (LINC) RNAs (LINC1335, LINC1333, and LINC1331) on chromosome 5. The leading SNPs at these loci were associated in the intensive glycemic control arm with 3.6- and 2.7-fold increases in risk of cardiovascular death per each copy of its allele (rs9299870, HR: 3.58; 95% CI 2.32–5.55 and rs57922, HR: 2.65 with 95%

CI 1.88–3.72, respectively). Combining the two variants together in a GRS ranging from 0 to 4 risk alleles, we found that those subjects carrying at least two risk alleles (around 30% of the population) had the double disadvantage of not deriving any benefit with respect to non-fatal myocardial infarction and experiencing a threefold increase in mortality when exposed to intensive glycemic control (red line on Fig. 6.8). By contrast, subjects with 0 or 1 risk allele experienced a reduction in non-fatal myocardial infarctions without any increase in mortality or even with a possible reduction in this outcome (green and yellow lines on Fig. 6.8). Similar effects were found in an observational cohort (the Joslin Kidney Study), in which only subjects carrying 0 or 1 risk alleles had a reduction of cardiovascular mortality when exposed to better glycemic control (defined as an HbA1c below the median level of 7.5%) [110]. These results were mechanistically enriched by the findings of an association between these variants and circulating fasting GLP-1 levels (glucagon-like peptide 1, active) [111]. Specifically, in the intensive glycemic control arm, subjects with 0 risk alleles for rs57922 (C/C homozygotes), i.e., those who derived the maximum cardiovascular benefits from intensive treatment, had a 22% increase in GLP-1 levels during follow-up, whereas subjects carrying two risk alleles (T/T homozygotes) had a 28% reduction in GLP-1 levels. These differences were not observed in the standard glycemic control arm, leading to a significant gene-by-intervention interaction. Altogether, these results suggest that a simple genetic test may allow the identification of those patients who might experience a cardiovascular benefit from more intense glycemic control than it is currently recommended (i.e., <6.0% vs. <6.5%). These data also suggest that those patients with detrimental cardiovascular response to intensive glycemic control might be identified by measuring GLP-1 levels after treatment intensification, and that these patients may especially benefit from the use of GLP1R agonists to lower their blood

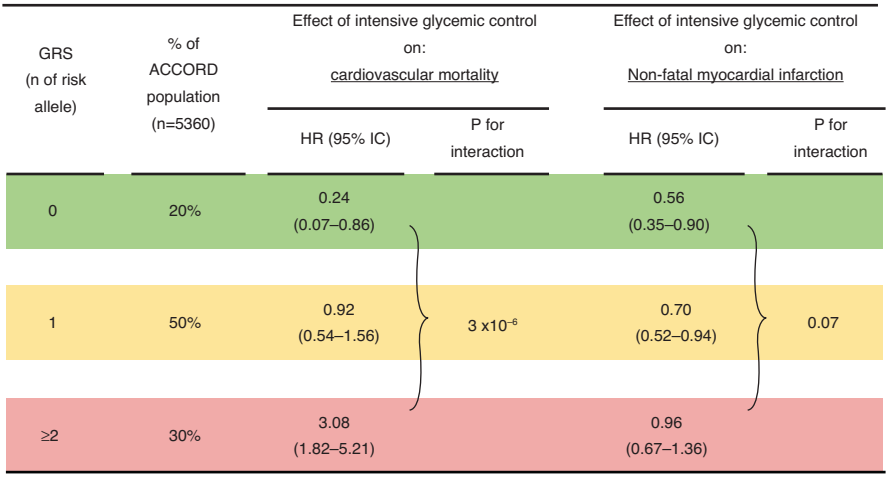

Fig. 6.8 Effect of intensive glycemic control on cardiovascular mortality and non-fatal myocardial infarction among participants in the ACCORD clinical trial stratified by a Genetic Risk Score derived from the combination of risk alleles at the 10q26 and 5q13 loci

glucose. These data require additional validation before getting to the clinic, especially with regard to non-white populations, since these were not included in the genetic studies of ACORD because of their small sample size. Nonetheless, these findings illustrate the potential for using genetic markers in a clinical setting.

Pharmacogenetic Studies on the Cardiovascular Effectiveness of Fenofibrate

Over the past few decades, several studies have investigated the cardiovascular effect of fibrates, including fenofibrate, in the general population and in particular in patients with type 2 diabetes [112–116]. Through the activation of the transcriptional factor peroxisome proliferator-activated receptor alpha (PPAR-alpha), fibrates improve lipid profile, and in particular, they contrast the so-called atherogenic dyslipidemia (defined by high triglycerides and low HDL-cholesterol levels)—a condition that is often present in patients with diabetes [117]. Fibrates, including fenofibrate, have also anti-inflammatory and anti-platelet actions that are independent from their lipid-lowering effect [118, 119]. However, despite these promising features, results from clinical trial on cardiovascular outcomes have been disappointing, in particular in those studies testing the efficacy of fibrates as adds-on to statin treatment [113, 114, 120], which have shown a heterogeneous response, with beneficial treatment only in the group of patients with atherogenic dyslipidemia [121–124]. Therefore, fibrates are not generally recommended by guidelines for cardiovascular prevention and might be considered only in those subjects with atherogenic dyslipidemia [66, 125]. Since several studies have shown that the lipid and anti-inflammatory response to fenofibrate might be partially genetically determined [126, 127], we tested whether genetic variants could also be used to identify subjects with diabetes having a better cardiovascular response to fenofibrate. To maximize our chances of success, we leveraged the many studies on fenofibrate and PPAR-alpha activation and followed a candidate-gene approach using the data from ACCORD-Lipid clinical trial [128, 129], in which more than 4000 patients with type 2 diabetes were randomized to fenofibrate or placebo on top of statin therapy, and for whom genetic data were available. First, we found that the cardiovascular effectiveness of fenofibrate was influenced by a common gain-of-function genetic variant (p.S447*) in the *LPL* gene, encoding for lipoprotein lipase (whose activity is enhanced by fenofibrate-induced PPAR-alpha activation [130, 131]), and already known to lower CAD risk [129, 132]. Specifically, we found that those subjects already carrying the allele increasing LPL activity did not derive any cardiovascular benefit from randomization to fenofibrate (RR 1.56; 95% CI 0.98–2.47), whereas all other subjects experienced a 19% risk reduction in the risk of major cardiovascular events (MACE) (RR 0.81%; 95% CI 0.66–1.00, p for interaction 0.01). This finding, given the only nominally significance of the negative interaction and the lack of additional evidence to date, should be considered as merely hypothesis generating. However, this observation was important as it suggested that genetic variants in the PPAR-alpha pathway could be used to identify subjects with better response to fenofibrate. This was confirmed in another study, in which we tested more than 400 common variants at the

PPARA locus, finding a variant (rs6008845) showing a study-wide significant interaction with fenofibrate on MACE ($p = 4 \times 10^{-4}$) [128]. This interaction was discovered in Whites patients, validated in African-Americans patients, and confirmed in observational cohorts. When all the observations from these different settings were combined together, they yielded a p value for interaction of 1×10^{-6}. The interaction was such that, as shown in Fig. 6.9 Panel (a), those subjects carrying the T/T genotype (about one third of the population included in ACCORD trial) had a 51% MACE risk reduction over a median follow-up of 4.7 years of treatments (HR 0.49, 95% CI 0.34–0.72), while subjects carrying other genotypes had no reduction in risk of MACE in response to this treatment. More importantly, the benefit of treatment with fenofibrate among those with T/T genotype was confirmed also in the subgroup of patients without atherogenic dyslipidemia, i.e., those for whom there was not current indication for treatment with fenofibrate. Based on these results, we estimated that the clinical benefit of fenofibrate, as assessed by the number of patients needed to be treated to avoid 1 MACE over the following 5 years (NNT), was similar among subjects without dyslipidemia but with the rs6008845 T/T genotype to that among subjects with atherogenic dyslipidemia. These results, while requiring further validation, clearly point to a pharmacogenetic approach towards optimal prescription of fenofibrate in patients with type 2 diabetes, which as shown in Fig. 6.9 Panel (b), would double the proportion of subjects who would benefit from this treatment.

Beyond the identification of a marker allowing the identification of subjects with better response to fenofibrate, these data have also provided important new insights

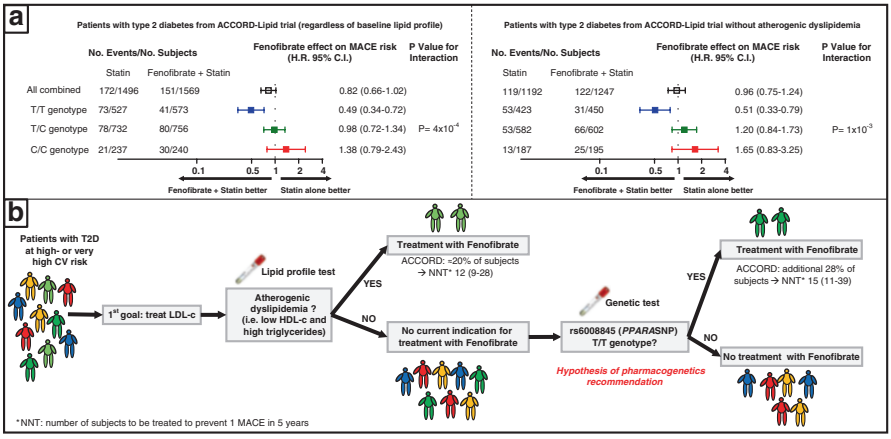

Fig. 6.9 Genetic variant (rs6008845) at the *PPARA* locus influencing the cardiovascular response to fenofibrate in ACCORD-Lipid. Panel (**a**): effectiveness of fenofibrate in reducing major cardiovascular events (MACE) risk in self-reported white patients from the ACCORD-Lipid trial. The results in the entire population are on the left and the results among participants without atherogenic dyslipidemia (i.e., those among whom fenofibrate is not currently recommended for cardiovascular prevention) are on the right. Panel (**b**): hypothesis of pharmacogenetic approaches allowing the identification of a larger proportion of patients deriving benefit from fenofibrate as compared to patients identified solely through lipid profile. (Adapted from Morieri et al. Diabetes 2020 [128])

into the mechanism of action of fenofibrate. First, the SNP modulating the response to fenofibrate, which is located ~25 kb from the *PPARA* starting site, was associated with PPAR-alpha expression in multiple tissues. Although the relevant tissue(s) involved in the genetic effect have not yet been identified, this supports the hypothesis that genetically determined PPAR-alpha expression (and activity) can influence the cardiovascular effectiveness of fenofibrate [128]. Second, we found that the differences in cardiovascular risk reduction across genotypes were not paralleled by differences in fenofibrate-induced changes in lipid profile as captured by HDL-cholesterol, triglycerides, or LDL-cholesterol levels. Although counterintuitive, these findings support those of a previous study showing that the lipid-lowering actions of fibrates explain only a small fraction of their cardiovascular effects (as small as 25% in the VA-HIT trial) [133] and that other metabolic effects of fibrates are probably involved. Indeed, in a small subset of ACCORD patients, we found that participants with the T/T genotype, i.e., those with a better cardiovascular response, had significantly lower levels of a pro-atherogenic chemokine (CCL11 or Eotaxin) after treatment with fenofibrate [128]. Whether these findings can be confirmed in larger populations, whether they explain the observed genetic effect, and what is the nature of the tissue(s) and cell type(s) involved in this genetic modulation is being currently investigated.

Conclusions and Future Directions

Over the past 15 years, there has been an exponential increase in our understanding of the genetic background of coronary artery disease in diabetes. Hundreds of CAD-associated loci have been discovered in the general population, and although most of them have yet unknown function, they have been found to increase CAD risk also in patients with diabetes. Genetic studies have provided clear validation of the long-standing hypothesis of a "common soil" between CAD and T2D, by identifying genetic variants, in particular those linked to insulin resistance, increasing the risk for both conditions. Genetic variants that increase CAD risk specifically in the presence of diabetes have also been identified and are currently under further investigation to develop new CVD-preventing treatments in diabetes. The combination of genetic variants into GRS's has been shown to improve prediction of future cardiovascular events that might be useful for research and clinical purposes. Most importantly, the discovery of genetic variants associated with CAD has allowed the design of new drugs (e.g., PCSK9 inhibitors), and is paving the way to genetic-guided approaches to prescribe cardiovascular prevention treatments more precisely and effectively.

Yet, this is most likely just the beginning of the use of genetic findings to improve treatment and prevention of CAD in patients with diabetes and in the general population. Despite these exciting results, the genetic analyses performed to date still have many limitations, which offer additional opportunities and point to new directions for future research. For instance, we are still far from being able to explain all the heritability and genetic susceptibility to CAD. The main reasons for this are the relatively small effects of individual variants and the need to apply stringent significance

threshold ($p < 5 \times 10^{-8}$) to avoid false-positive findings. The combination of these factors has translated into limited statistical power, despite the relatively large sample size of the studies conducted thus far. Further increasing sample size, as it is being done by initiatives such as the UK Biobank, will provide a better definition of the association of the CAD variants identified to date and will likely lead to the identification of many other ones, including those with a low frequency, which have been thus far overlooked. Larger studies will also allow evaluation of possible gene-by-gene interactions (epistasis) affecting the risk of CAD. Indeed, we are currently estimating the genetically determined risk conferred by variants considered individually, whereas the effects of some of them might depends on the presence of other variants. Testing this hypothesis on genome-wide scale, including millions of variants, will require very large sample sizes (i.e., millions of subjects) to achieve adequate power to reject the null hypothesis of no epistasis [134]. At the same time, sample sizes will need to be increased without sacrificing the quality on the phenotypic information, making sure, for instance, that diabetes is properly defined and precedes the onset of CAD in order to avoid the problems discussed in the section on "Development of New Preventive Interventions". Finally, current studies are still mainly focused on European populations with inadequate representation of individuals of other ancestries. In fact, the majority of CAD loci discovered in the general population has been derived from studies of non-Hispanic whites, and GRS based on these variants show a poor performance in other racial groups. This problem has been reported not only in the general population [12] but also among subjects with diabetes. For instance, the two GRS for CAD developed in the Look-Ahead study and the ACCORD trial were not associated with increased risk in African-American subjects with type 2 diabetes [17, 19]. There is therefore the need to develop GRS including ancestry-specific loci and variants [12]. Moreover, as it has been the case for studies of the genetics of type 2 diabetes [22], these multi-ancestry approaches may foster the discovery of additional genetic variants associated with CAD. A case in point is the identification of the loss-of-function mutation of PCKS9 in African-American individuals, which prompted the development of PCSK9 inhibitors. Overcoming these challenges will be essential to continue the path towards a fast and highly effective translation of genetic findings into better strategies to prevent CAD and decrease the burden of this health problem among patients with diabetes.

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