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ORIGINAL CONTRIBUTION

Flavonoid intake from fruit and vegetables during adolescence is prospectively associated with a favourable risk factor profile for type 2 diabetes in early adulthood

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Abstract

Purpose Flavonoid consumption during adolescence could contribute to preventing adult onset of type 2 diabetes mellitus. We investigated the prospective association between habitual intake of flavonoids from fruit and vegetables (FlavFV) during adolescence and risk markers of type 2 diabetes in early adulthood.

Methods This analysis included participants of the DONALD Study, who had provided a fasting blood sample in adulthood (18–39 years), data on FlavFV-intake during adolescence (females: 9–15 years, males: 10–16 years) and relevant covariates. Habitual FlavFV-intake was either estimated using repeated 3-day weighed dietary records ($n = 268$), or the validated biomarker hippuric acid (uHA)-excretion in repeated 24-h urine samples ($n = 241$). Multivariable linear regressions were performed to analyse the prospective associations of FlavFV or uHA with homeostasis model assessment insulin sensitivity (HOMA2-%S), hepatic steatosis index (HSI), fatty liver index (FLI) and a pro-inflammatory score.

Results Higher FlavFV-intake was independently related to higher HOMA2-%S among females ($P_{trend} = 0.03$), but not among males. Both FlavFV-intake and uHA-excretion were inversely associated with HSI ($P_{trend} < 0.0001$ and $P_{trend} = 0.02$, respectively) and the pro-inflammatory score ($P_{trend} = 0.02$ and $P_{trend} = 0.008$, respectively), but not with FLI.

Conclusions Our data indicate that flavonoid consumption from fruit and vegetables during adolescence is associated with a favourable risk factor profile for type 2 diabetes in early adulthood.

Keywords 24-Hour urinary hippuric acid excretion · Chronic subclinical inflammation · Flavonoids from fruit and vegetables · Homeostasis model assessment of insulin sensitivity · Indices of hepatic steatosis · Prospective

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Introduction

Several lines of evidence support a benefit of flavonoids for prevention of type 2 diabetes mellitus and its risk factors such as hepatic steatosis and chronic subclinical inflammation. A meta-analysis of prospective studies indicates a reduced type 2 diabetes risk among those in the highest category of total flavonoid intake [1]. Randomized controlled trials (RCTs) administering either anthocyanins or an anthocyanin-rich drink demonstrate improved aminotransferase activities [2, 3], i.e. surrogate markers of hepatic fat accumulation. A meta-analysis of RCTs reported that increased flavonoid and berry intake reduced systemic concentrations of inflammatory biomarkers [4, 5].

Available evidence predominantly stems from studies among adults. However, obesity, insulin resistance, hepatic fat accumulation and chronic subclinical inflammation emerge already during adolescence [6, 7], and may track into adulthood thus contributing to the development of type 2 diabetes [8] and non-alcoholic fatty liver disease [6]. Accordingly, adolescence, which is characterized by substantial hormonal, metabolic and lifestyle changes, may be considered a critical period [9] for later cardiometabolic diseases, but also represents a window of opportunity for prevention by lifestyle modifications including flavonoid consumption.

In terms of public health, the overall preventive potential of total flavonoids from fruit and vegetables (including juices, FlavFV) is of interest rather than the role of individual flavonoid subclasses. In addition, fruit and vegetables usually represent the main flavonoid source in the diet of adolescents (contributing approximately 75% to total flavanol intake among German adolescents [10]). Further, the focus on FlavFV allows elucidation whether flavonoids from these sources are mechanistically responsible for part of the health benefits ascribed to fruit and vegetables. Cocoa products are also generally regarded an important flavonoid source, however, they should be judged differently from a public health perspective since they commonly also provide other less favourable nutrients (e.g. added sugar). Since flavonoid intake estimation by dietary instruments is prone to bias, it should ideally be combined with a validated intake biomarker [11]. We have recently validated the concentration biomarker [12] 24-h urinary hippuric acid (uHA) excretion, which proved to be valuable for categorization of healthy adolescents into high, medium and low consumers of FlavFV (Spearman correlation coefficient $r = 0.53$, $P < 0.0001$; 83% versus 4% of the subjects classified into same/adjacent versus opposite quartiles, respectively) [13]. So far, no study has employed a comprehensive estimation of flavonoid intake on both the diet and biomarker level. Moreover, no study investigated the long-term relevance of flavonoid intake in youth for adult metabolic health.

Therefore, this study addresses the hypothesis that FlavFV habitually consumed during adolescence (assessed on a dietary and a biomarker level) beneficially affect risk markers of type 2 diabetes in adulthood, i.e. insulin sensitivity, indices of hepatic steatosis and inflammatory biomarkers.

Subjects and methods

Study population

The present analysis is based on data from the Dortmund Nutritional and Anthropometric Longitudinally Designed Study (DONALD Study), an ongoing, open-cohort study conducted in Dortmund, Germany, since 1985 [14]. Briefly, approximately 35–40 healthy infants are newly recruited each year to collect detailed data on diet, growth, and metabolism from infancy to adulthood. During childhood, participants are invited annually until adulthood. Since 2005, participants are invited for follow-up in adulthood including blood withdrawal every 5 years. The study was approved by the Ethics Committee of the University of Bonn (Germany) and conducted according to the guidelines of the Declaration of Helsinki. Written parental and adult participants' informed consent is obtained for all examinations.

At the time of this analysis, 397 participants had provided a fasting blood sample in adulthood (18–39 years) for the measurement of risk markers of type 2 diabetes and fulfilled the following eligibility criteria: singletons, born at term (37 to < 43 gestation weeks) with normal birth weight. To estimate habitual intake of FlavFV during adolescence (females: 9–15 years, males: 10–16 years), participants additionally had to have provided either at least two 3-day weighed dietary records ($n = 276$ after exclusion of 19 participants with ≥ 50% implausible dietary records [15]) or at least two complete 24-h urine samples ($n = 247$) for the measurement of uHA-excretion, a validated biomarker of FlavFV intake. Finally, anthropometric measurements from adolescence and adulthood, and data on relevant covariates were required, resulting in analysis samples of 268 participants for the dietary level and 241 participants for the urinary biomarker level (with $n = 230$ providing both dietary and urinary data).

Smaller sample sizes for fasting glucose, insulin and HOMA2-%S ($n = 256$ in dietary sample, 235 in urinary sample) and inflammation ($n = 265$ in dietary sample, 238 in urinary sample) resulted from exclusion of participants with missing analytes due to insufficient amounts of blood or fasting glucose concentrations below the threshold for calculation of HOMA2-%S (reflecting hypoglycaemia).

Dietary assessment

Dietary intake is assessed annually by 3-day weighed dietary records according to instructions by trained dieticians, which includes weighing and recording of all consumed foods and beverages as well as leftovers to the nearest g or alternatively semi-quantitative recording, if weighing is not possible. Nutrient intake (without supplements) was determined using the continuously updated in-house nutrient database LEBTAB [16].

As previously described [13], dietary FlavFV-intake was estimated after assignment of flavonoid contents from USDA databases to the recorded food items. Assignment was performed on the recipe level following a standardized procedure. The impact of food processing on flavonoid contents was accounted for by retention and/or yield factors. For the present analysis, individual FlavFV-intake was calculated as the sum of the assigned flavonoid (i.e. flavones, flavonols, flavan-3-ol-monomers, flavanones and anthocyanidins) and proanthocyanidin (i.e. flavan-3-ol-dimers to polymers) content (excluding isoflavones). Subsequently, individual nutrient and FlavFV-intakes were averaged over the three recorded days. To describe the participants' habitual intake, an individual mean was calculated from all available records during adolescence (2 to 7 records per person, mean = 6).

Urine collection and analysis

Participants are requested to collect 24-hour urine annually according to instructions. All micturitions from the 24-h sampling period were collected in provided Extran-cleaned (Extran, MA03, Merck Darmstadt, Germany) preservative-free 1-L plastic containers and stored immediately at ≤ − 12 °C. After transport to the study centre, the samples were stored at ≤ − 20 °C until analysed. Completeness of 24-h urine collections was determined by creatinine excretion, which was measured photometrically by the kinetic Jaffé procedure on a creatinine analyser (Beckman-2; Beckman Instruments) [17].

As described previously, uHA (in triplicate) was measured photometrically with modifications of the method according to Tomokuni and Ogata [13, 18]. Intra- and inter-assay coefficients of variation (CV) were 3.8 and 6.3%, respectively. An individual mean of uHA-excretion was calculated from all available 24-h urine samples during adolescence (2 to 5 samples per person, mean = 4.5) to reflect habitually ingested flavonoids.

Blood sampling and analysis

Venous blood samples were drawn after an overnight fast, centrifuged at 4 °C within 15 min and stored at − 80 °C. Eleven participants had provided two blood samples between 2005 and 2015, of which the latest sample was chosen, unless sample material was not sufficient for all blood analyses. The following blood analytes were measured at the German Diabetes Center: plasma activities of liver enzymes, plasma triglycerides and plasma high-sensitivity C-reactive protein (hsCRP) using the Roche/Hitachi Cobas c311 analyser (Roche diagnostics, Mannheim, Germany), plasma high-sensitivity interleukin (IL)-6 with the Human IL-6 Quantikine HS, plasma adiponectin with the Human Total Adiponectin/Acrp30 Quantikine ELISA and serum leptin with the Leptin Quantikine ELISA kits all from R&D Systems (Wiesbaden, Germany), serum IL-18 with the Human IL-18 ELISA kit from MBL (Nagoya, Japan), and plasma chemerin with the Human Chemerin ELISA kit from BioVendor (Brno, Czech Republic). Intra- and inter-assay CV for hsCRP, IL-6, IL-18, chemerin, and adiponectin were 1.0 & 2.6%, 7.2 & 11.8%, 3.7 & 7.1%, 3.4 & 6.4%, and 3.8 & 8.0%, respectively. Plasma concentrations of insulin were analysed at the Laboratory for Translational Hormone Analytics of the University of Giessen using an immunoradiometric assay (IRMA, DRG Diagnostics, Marburg, Germany) and the updated HOMA2-%S was calculated [19]. Indices of hepatic steatosis were calculated as follows [20, 21]:

$$\begin{aligned}\text{HSI} = {} & 8 \times (\text{Alanine} - \text{aminotransferase/aspartate} \\ & - \text{aminotransferase}) + \text{BMI} (+ 2 \text{ if diabetes mellitus}; \\ & + 2 \text{ if female}),\end{aligned}$$

$$\begin{aligned}\text{FLI} = {} & e^x/(1 + e^x) \times 100 \\ \text{with } x = {} & 0.953 \times \ln(\text{triglycerides (mg /dL)}) \\ & + 0.139 \times \text{BMI} + 0.718 \\ & \times \ln(\gamma\text{-glutamyltransferase (U /L)}) \\ & + 0.053 \times \text{waist circumference (cm)} \\ & - 15.745.\end{aligned}$$

A pro-inflammatory score—assumed to be more predictive of inflammation than single markers [22]—was obtained as follows: (1) standardization of each inflammatory parameter (hsCRP, IL-6, IL-18, chemerin, leptin, adiponectin) by sex (mean = 0, SD = 1), (2) assignment of a minus sign to the anti-inflammatory parameter adiponectin to align its impact with the pro-inflammatory parameters, and (3) averaging all.

Anthropometric measurements

Anthropometric measurements were taken by trained nurses according to standard procedures. Standing height was measured to the nearest 0.1 cm (digital stadiometer: Harpenden Ltd., Crymych, UK) and body weight to the nearest 0.1 kg (electronic scale: Seca 753E, Seca Weighing and Measuring Systems, Hamburg, Germany).

From these measures the participants' body surface area (BSA) [13], BMI SD scores (sex- and age-specifically standardized according to German references [23]) as well as overweight during adolescence [24] were calculated. Waist circumference was measured at the midpoint between lower rip and iliac crest to the nearest 0.1 cm. Average intra- and inter-observer CV obtained from annual quality checks (2010 to 2014) for biceps, triceps, subscapular, and suprailiacal skinfolds were 10.4 & 10.9%, 5.3 & 3.3%, 6.1 & 4.1%, and 6.9 & 7.5%, respectively.

Assessment of further covariates

Further covariates were collected on the child's admission to the DONALD Study or periodically at follow-up visits. The child's birth characteristics were retrieved from the "Mutterpass" (a German standardized pregnancy document). Parental and familial information was assessed by anthropometrical and medical examinations of the child's parents and interviews about disease history and socioeconomic status. The participant's smoking status and physical activity was assessed by questionnaires.

Statistical analysis

Characteristics of the study population are presented as mean ± SD or median (25th, 75th percentile) for continuous variables and as absolute (relative) frequencies for categorical variables. Associations between outcome variables were calculated as Pearson correlation.

To achieve normal distribution in outcome variables we used $\log_e$ or reciprocal transformations. Individual outliers which substantially interfered with normal distribution of the residuals or regression modelling (i.e. if associations only appeared when outliers were included), were winsorized, i.e. outliers were replaced by the sex-specifically closest value fitting the distribution. Winsorization concerned < 4% of the data for FLI, pro-inflammatory score, glucose, hsCRP, IL-6, or adiponectin. Before calculating the individual means from available records or urines during adolescence, dietary variables were energy-adjusted by the residual method and standardized by age group and sex to account for age- and sex-dependent intake differences. Analogously, uHA was adjusted for BSA (standardized by age group and sex), since it is closely related with individual body size-dependent glomerular filtration rate [25]. As such it codetermines uHA and can be used as a proxy measure for energy requirements, which was applied because energy intake was not available for the entire urinary sample. To achieve normally distributed residuals square root (FlavFV) and $\log_e$ transformations (other dietary variables and uHA) were applied.

Prospective associations between FlavFV or uHA during adolescence and risk markers of type 2 diabetes in early adulthood were analysed by multivariable linear regression models, using the transformed variables as explained above. Formal interaction analyses indicated sex-interactions for HOMA2-%S and insulin on the dietary level ($P_{interaction}$=0.04); therefore, sex-stratified analyses were performed for the parameters of insulin sensitivity (glucose, insulin and HOMA2-%S) on both the dietary and the biomarker level to allow comparability.

Initial regression models (model A) included the predictors FlavFV or uHA as well as age at blood withdrawal and sex (in pooled analyses). Adjusted models (model B) were constructed by individual examination of potential influencing covariates and hierarchical inclusion of those which substantially modified the predictor–outcome associations (≥ 10%) or significantly predicted the outcome. Potential confounding covariates considered in the hierarchical approach were (1) early life factors [birth weight (g), gestational age (week), maternal age at birth (year) and gestational weight gain (kg)], (2) socioeconomic factors and parental health status [smokers in the household (yes/no), paternal school education ≥ 12 years (yes/no), parental overweight (BMI ≥ 25 kg/m^2 yes/no) and parental history of diabetes (yes/no)], (3) predictor-specific adolescent data (BMI-SD score and energy-adjusted dietary intake (protein, total and saturated fat, carbohydrate, added sugar as well as fibre, fructose, potassium, magnesium and vitamin C from fruit and vegetables adjusted for FlavFV intake) in models with the dietary predictor FlavFV, and BSA in models with the urinary predictor uHA). In conditional models we additionally included adult waist circumference (cm) to investigate whether observed associations were partly attributable to body composition in adulthood. To retain comparability of results, models were adjusted identically for closely related outcomes (parameters of insulin sensitivity (fasting glucose, fasting insulin, HOMA2-%S), parameters of hepatic steatosis (HSI and FLI), and inflammatory parameters (pro-inflammatory score, hsCRP, IL-6, IL-18, chemerin, leptin, adiponectin)). Results from regression analyses are presented as adjusted least-square means (95% CI) by tertiles of the respective predictor with *P* values from models with the predictors as continuous variables.

To elucidate whether the sum of flavonoids from fruits and vegetables (FlavFV) is indeed the most relevant predictor during adolescence, comparative analyses were conducted using total flavonoids (sum of flavonoids from fruits, vegetables, juices, cocoa, tea, coffee, nuts and legumes), flavonoids from cocoa, nuts and the individual sources of FlavFV (i.e. fruits, vegetables and juices each). Due to very low consumption levels (see Table 1) and percentages of consumers, flavonoids from tea and legumes were not considered individually.

Sensitivity analyses in subsamples of participants who had provided certain data in adulthood were performed by

Table 1 Baseline characteristics of the DONALD participants in adolescence (males: 10–16 years, females: 9–15 years): anthropometry, dietary and urinary data as well as early life and socioeconomic factors

	n	Dietary sample	*n*	Urinary sample
Females/males (%)	268	52/48	241	51/49
Data from adolescence				
Age (year)	268	12.1 (12.0, 13.0)	241	12.3 (11.8, 13.0)
Anthropometry, dietary and urinary data				
BMI-SD score	268	− 0.19 ± 0.85	241	− 0.13 ± 0.88
BMI (kg/m^2)	268	18.6 (16.8, 20.2)	241	18.9 (16.9, 20.6)
BSA (m^2)	268	1.4 (1.3, 1.6)	241	1.5 (1.3, 1.6)
Overweight (%)[a]	268	22	241	26
Total energy (MJ/d)	268	8.0 (6.9, 9.0)		
Fat (%en)	268	35.6 ± 4.0		
SFA (%en)	268	15.7 ± 2.1		
Protein (%en)	268	13.0 ± 1.6		
Carbohydrate (%en)	268	51.2 ± 4.3		
Fibre (g/MJ)	268	2.45 (2.15, 2.78)		
Fruit and vegetables (g/d)[b]	268	435 (324, 573)		
Total flavonoids (mg/d)	268	204 (160, 252)		
From fruit and vegetables (FlavFV, mg/d)[b]	268	131 (88, 179)		
From fruit (mg/d)	268	75 (46, 119)		
From vegetables (mg/d)	268	9 (5, 16)		
From juices (mg/d)	268	35 (19, 56)		
From cocoa (mg/d)	268	39 (26, 58)		
From nuts (mg/d)	268	12 (7, 22)		
From legumes (mg/d)	268	0.1 (0.0, 0.4)		
From tea (mg/d)	268	0.7 (0.0, 11.3)		
Urinary hippuric acid (uHA, mmol/24 h)			241	2.8 (2.4, 3.4)
Early life and socioeconomic factors				
Birth weight (g)	268	3450 (3130, 3800)	241	3470 (3130, 3810)
Gestational age (week)	268	40 (39, 41)	241	40 (39, 41)
Maternal gestational weight gain (kg)	268	12 (10, 15)	241	12 (10, 15)
Maternal age at birth (year)	268	30.3 (28.1, 33.2)	241	30.3 (28.1, 33.3)
Paternal school education ≥ 12 years (%)	258	60	236	58

Values are means ± SD, medians (25th, 75th percentile) or relative frequencies. *BSA* body surface area, *DONALD* Dortmund Nutritional and Anthropometric Longitudinally Designed

[a]Defined according to age- and sex-specific cut points of the International Obesity Task Force (Cole et al. 2000 [24])

[b]Including juices

additional inclusion of the following variables: (a) adult FlavFV-intake ($n = 239$ in dietary sample), (b) adult smoking status ($n = 247$ in dietary sample and $n = 219$ in urinary sample), (c) adult alcohol consumption (for liver-associated outcomes only; $n = 239$ in dietary sample and $n = 212$ in urinary sample) and (d) levels of adult physical activity (low/medium/high; $n = 267$ in dietary sample and $n = 240$ in urinary sample).

The SAS statistical software package version 9.2 (SAS Institute Inc., Cary, NC) was used for all statistical analyses. The significance level was set at $P < 0.05$.

Results

Characteristics of the participants at baseline and at follow-up are presented in Tables 1 and 2, respectively. Participants were characterized by a BMI-SD score below average, and an above-average socioeconomic status, as indicated by the high percentage of participants' fathers with a high educational level. The median follow-up time between the mean age during adolescence and adulthood was 9 and 8.6 years in the dietary and urinary sample, respectively.

In dietary ($n = 252$) and urinary samples ($n = 231$), the correlation between the two indices of hepatic steatosis

Table 2 Characteristics of the DONALD participants at follow-up in early adulthood: anthropometry, dietary and lifestyle data and risk markers of type 2 diabetes

	n	Dietary sample	*n*	Urinary sample
Adult age (year)	268	21.1 (18.1, 24.2)	241	20.9 (18.1, 23.9)
Anthropometry, dietary and lifestyle data				
BMI (kg/m^2)	268	22.2 (20.7, 24.9)	241	22.4 (20.8, 25.0)
Waist circumference (cm)	268	75.9 (70.7, 81.3)	241	76.2 (71.0, 81.8)
Total energy (MJ/d)	239	9.1 (7.7, 11.1)		
Fruit and vegetables (g/d)[a]	239	434 (277, 661)		
Flavonoids from fruit and vegetables (mg/d)[a]	239	106 (60, 175)		
Alcohol (g/d)	239	0.39 (0.06, 6.10)		
Current smoking (%)	247	27	219	28
Risk markers of type 2 diabetes				
Glucose (mmol/L)	256	5.28 (5.01, 5.61)	235	5.28 (5.01, 5.61)
Insulin (pmol/L)	256	67.8 (53.7, 87.2)	235	68.4 (54.4, 88.2)
HOMA2-%S	256	77 (61, 99)	235	77 (60, 98)
TG (mmol/L)	268	1.04 (0.80, 1.36)	241	1.04 (0.80, 1.38)
ALT (U/L)	268	16.2 (13.1, 21.1)	241	15.9 (12.9, 20.9)
AST (U/L)	268	21.1 (18.6, 24.6)	241	20.9 (18.5, 24.3)
GGT (U/L)	268	14.2 (11.0, 19.0)	241	14.1 (10.9, 19.0)
HSI	268	29.8 (27.8, 33.2)	241	29.9 (27.8, 33.4)
FLI	268	7.4 (4.6, 15.9)	241	8.0 (4.7, 17.8)
hsCRP (mg/L)	265	0.8 (0.4, 1.8)	238	0.9 (0.4, 1.9)
IL-6 (pg/mL)	265	0.68 (0.48, 1.03)	238	0.68 (0.48, 1.06)
IL-18 (pg/mL)	265	250 (206, 308)	238	250 (205, 308)
Chemerin (ng/mL)	265	154 (134, 176)	238	155 (135, 177)
Leptin (ng/mL)	265	6.62 (2.67, 12.75)	238	6.95 (2.44, 13.24)
Adiponectin (µg/mL)	265	7.74 (5.39, 10.58)	238	7.65 (5.48, 10.58)
Inflammatory score	265	− 0.11 (− 0.38, 0.34)	238	− 0.12 (− 0.41, 0.32)

Values are means ± SD, medians (25th, 75th percentile) or relative frequencies

ALT alanine-aminotransferase, *AST* aspartate-aminotransferase, *DONALD* Dortmund Nutritional and Anthropometric Longitudinally Designed, *FLI* fatty liver index, *GGT* γ-glutamyltransferase, *HOMA2-%S* updated homeostasis model assessment of insulin sensitivity, *hsCRP* high-sensitivity C-reactive protein, *HSI* hepatic steatosis index, *TG* triglycerides

[a]Including juices

($r = 0.7$, $P < 0.0001$), between both indices and the pro-inflammatory score ($r = 0.5$, $P < 0.0001$) and between HOMA2-%S and the aforementioned outcomes ($r = -0.2$, $P < 0.003$)

Adolescent flavonoid intake and adult insulin sensitivity

A higher adolescent FlavFV-intake was independently related to higher adult HOMA2-%S and lower fasting insulin among females (P_{trend}=0.032 and P_{trend}=0.029, respectively; Table 3, model B), but not among males ($P_{interaction} = 0.04$). Similar, but non-significant trends were also observed on the biomarker level among females only ($P_{trend} = 0.1$ for fasting insulin and HOMA2-%S; Table 3, model B). Additional inclusion of adult waist circumference did not affect the results (Table 3, conditional model).

Adolescent flavonoid intake and adult hepatic steatosis

Both a higher adolescent FlavFV-intake and a higher uHA-excretion were independently associated with a lower adult HSI (Fig. 1a, b). Similar, albeit non-significant inverse associations were observed with FLI (Fig. 1c, d).

Table 3 Sex-stratified prospective associations of dietary flavonoid intake from fruit and vegetables (FlavFV) and urinary hippuric acid (uHA) excretion during adolescence with markers of insulin sensitivity in early adulthood (FlavFV: 125 males, 131 females; uHA: 119 males, 116 females)

	Tertiles of FlavFV intake during adolescence ($n = 256$)				Tertiles of uHA excretion during adolescence ($n = 235$)			
	T1	T2	T3	P_{trend}	T1	T2	T3	P_{trend}
Females								
FlavFV (mg/d)[a]	74 (60, 88)	133 (113, 146)	197 (173, 224)		87 (62, 139)	114 (88, 161)	159 (138, 185)[b]	
uHA (mmol/24 h)[a]	2.4 (2.0, 2.6)	2.6 (2.3, 3.0)	2.8 (2.5, 3.5)[b]		2.1 (1.8, 2.5)	2.5 (2.4, 2.8)	3.3 (2.8, 4.1)	
Glucose (mmol/L)								
Model A	93.1 (91.1 to 95.2)	92.8 (90.8 to 94.8)	92.9 (90.9 to 94.9)	0.9	93.3 (91.1 to 95.5)	93.1 (91.0 to 95.3)	92.6 (90.5 to 94.8)	0.6
Model B	93.0 (90.9 to 95.1)	92.9 (90.9 to 94.9)	92.9 (90.9 to 95.0)	> 0.9	92.7 (90.6 to 94.9)	93.2 (91.1 to 95.3)	93.1 (91.0 to 95.3)	0.8
Conditional model	92.8 (90.8 to 94.9)	93.0 (91.0 to 95.0)	93.0 (91.0 to 95.0)	0.9	92.7 (90.6 to 94.9)	93.2 (91.1 to 95.3)	93.2 (91.0 to 95.3)	0.9
Insulin (pmol/L)								
Model A	77.9 (70.7 to 85.9)	68.4 (62.2 to 75.3)	67.6 (61.4 to 74.4)	0.048	78.8 (71.0 to 87.5)	70.4 (63.5 to 78.0)	70.3 (63.4 to 77.9)	0.085
Model B	78.1 (71.0 to 85.9)	69.1 (63.1 to 75.7)	66.8 (60.9 to 73.2)	0.029	76.9 (69.4 to 85.2)	71.3 (64.5 to 78.9)	71.0 (64.2 to 78.6)	0.1
Conditional model	77.7 (70.7 to 85.4)	69.4 (63.4 to 76.0)	66.8 (61.0 to 73.2)	0.031	76.5 (69.1 to 84.6)	71.2 (64.5 to 78.6)	71.5 (64.7 to 79.0)	0.2
HOMA2-%S								
Model A	68.6 (62.3 to 75.5)	78.2 (71.1 to 85.9)	79.1 (72.0 to 87.0)	0.050	67.8 (61.2 to 75.2)	75.8 (68.5 to 84.0)	76.4 (69.0 to 84.6)	0.065
Model B	68.5 (62.4 to 75.2)	77.4 (70.8 to 84.7)	80.0 (73.0 to 87.6)	0.032	69.6 (62.9 to 77.0)	74.9 (67.8 to 82.7)	75.5 (68.3 to 83.4)	0.1
Conditional model	68.9 (62.7 to 75.6)	77.1 (70.5 to 84.3)	79.9 (73.0 to 87.4)	0.034	70.0 (63.4 to 77.3)	75.0 (68.0 to 82.7)	74.9 (67.9 to 82.7)	0.1
Males								
FlavFV (mg/d)[a]	71 (57, 90)	130 (116, 153)	211 (187, 257)		105 (56, 143)	132 (104, 162)	175 (117, 232)[3]	
uHA (mmol/24 h)[a]	2.5 (2.0, 3.2)	3.1 (2.7, 3.4)	3.9 (3.1, 4.7)[3]		2.4 (2.0, 2.6)	3.1 (2.8, 3.3)	3.9 (3.4, 4.7)	
Glucose (mmol/L)								
Model A	99.2 (96.8 to 101.7)	98.2 (95.8 to 100.6)	98.8 (96.4 to 101.3)	0.5	98.4 (96.0 to 100.9)	96.9 (94.6 to 99.4)	99.5 (97.0 to 102.0)	0.6
Model B	99.5 (96.9 to 102.1)	98.1 (95.6 to 100.6)	98.7 (96.3 to 101.2)	0.4	99.1 (96.6 to 101.7)	96.5 (94.2 to 99.0)	99.2 (96.8 to 101.7)	0.9
Conditional model	99.5 (97.0 to 102.1)	97.9 (95.4 to 100.4)	98.8 (96.4 to 101.4)	0.4	99.1 (96.6 to 101.7)	96.4 (94.0 to 98.8)	99.3 (96.9 to 101.8)	0.8
Insulin (pmol/L)								
Model A	62.7 (55.7 to 70.7)	65.7 (58.4 to 73.9)	71.3 (63.4 to 80.2)	0.4	65.8 (58.0 to 74.6)	70.2 (62.0 to 79.5)	63.7 (56.3 to 72.1)	0.6
Model B	63.4 (55.9 to 71.8)	65.4 (58.0 to 73.7)	70.9 (62.9 to 80.0)	0.5	67.1 (59.0 to 76.2)	70.0 (61.7 to 79.4)	62.7 (55.4 to 71.1)	0.5
Conditional model	64.0 (56.9 to 71.9)	63.3 (56.6 to 70.9)	72.5 (64.8 to 81.2)	0.4	67.1 (59.5 to 75.6)	68.8 (61.2 to 77.4)	63.8 (56.8 to 71.7)	0.5
HOMA2-%S								
Model A	83.3 (74.0 to 93.7)	80.1 (71.2 to 90.0)	73.9 (65.8 to 83.1)	0.4	79.6 (70.3 to 90.2)	75.3 (66.6 to 85.1)	82.3 (72.8 to 93.0)	0.6
Model B	82.4 (72.8 to 93.3)	80.4 (71.4 to 90.6)	74.3 (65.9 to 83.7)	0.6	78.0 (68.7 to 88.5)	75.6 (66.8 to 85.7)	83.6 (73.9 to 94.6)	0.5

Table 3 (continued)

	Tertiles of FlavFV intake during adolescence ($n = 256$)				Tertiles of uHA excretion during adolescence ($n = 235$)			
	T1	T2	T3	P_{trend}	T1	T2	T3	P_{trend}
Conditional model	81.6 (72.7 to 91.7)	83.0 (74.2 to 92.8)	72.7 (65.0 to 81.3)	0.4	78.0 (69.3 to 87.7)	76.9 (68.5 to 86.4)	82.2 (73.2 to 92.3)	0.5

Values are adjusted least-squares means (95% CIs) unless otherwise indicated. Linear trends (P_{trend}) were obtained in sex-stratified linear regression models with the predictor FlavFV or uHA as a continuous variable. Model A with predictor FlavFV, adjusted for adult age at blood withdrawal, and energy intake (residuals). Model A with predictor uHA, adjusted for adult age at blood withdrawal, and BSA (residuals). Model B with predictor FlavFV, additionally adjusted for maternal gestational weight gain, maternal age at birth, paternal education, and adolescent BMI-SD score. Model B with predictor uHA, additionally adjusted for maternal gestational weight gain, maternal age at birth, paternal education, and adolescent BSA. Conditional model, additionally adjusted for adult waist circumference. Transformations of variables for analysis: $\log_e$ for all markers of insulin sensitivity and uHA, square root for FlavFV. *FlavFV* dietary flavonoids from fruit and vegetables including juices, *HOMA2-%S* updated homeostasis model assessment of insulin sensitivity, *uHA* urinary hippuric acid

[a]Values are unadjusted medians (25th, 75th percentile)

[b]In reduced samples providing both 3-d weighed dietary records and 24-h urines during adolescence ($n = 111$ males, $n = 113$ females)

Adolescent flavonoid intake and adult chronic subclinical inflammation

A higher FlavFV-intake and a higher uHA-excretion during adolescence were also independently related to a lower adult pro-inflammatory score (Fig. 2a, b). Analyses of individual inflammatory parameters indicated a strong inverse association of adolescent FlavFV-intake and uHA-excretion with adult leptin in particular (Online Resource Supplementary Table 1). While the consideration of adult waist circumference did not affect the results for leptin (conditional model), it attenuated the association between FlavFV-intake – albeit not uHA-excretion – and the pro-inflammatory score ($P_{trend} = 0.06$).

Total flavonoids and flavonoids from specific sources

In comparative analyses total flavonoids were associated with HSI and the pro-inflammatory score with effect sizes comparable to those observed with FlavFV, however, no relation emerged with HOMA2-%S among females or males (Online Resource Supplementary Table 2). Separate consideration of flavonoids from fruits, vegetables and juices revealed that the direction of the associations was generally comparable to that seen with the sum of the three (i.e. our main exposure), however, consistently significant (or trend) associations were confined to flavonoids contributed by fruit. Separate consideration also confirmed the absence of an association with HOMA2-%S among males; instead flavonoids from cocoa seem to be of interest.

Sensitivity analyses

Sensitivity analyses in subsamples of participants who had provided additional data on adult smoking status, adult FlavFV-intake, adult alcohol consumption or adult physical activity yielded largely similar results.

Discussion

This prospective study shows that a higher habitual flavonoid intake from fruit and vegetables during adolescence is associated with a more favourable profile of risk factors for type 2 diabetes in early adulthood. This was consistently seen on the diet and biomarker level for both hepatic steatosis and inflammatory markers, while benefits for insulin sensitivity were only observed on the diet level

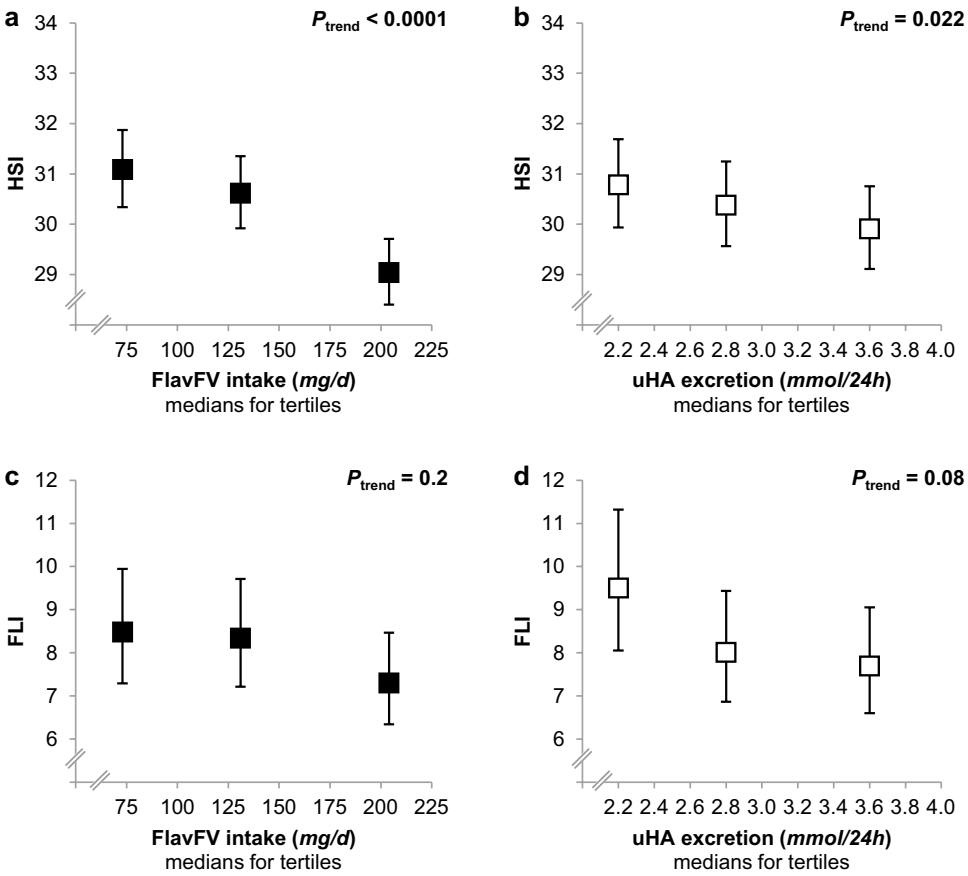

Fig. 1 HSI (**a, b**) and FLI (**c, d**) in early adulthood by tertiles of dietary flavonoid intake from fruit and vegetables (FlavFV; **a, c**; $n = 268$) and urinary hippuric acid (uHA; **b, d**; $n = 241$) during adolescence. Data are geometric means and 95% CI adjusted for sex, adult age at blood withdrawal, gestational age, birth weight, maternal gestational weight gain, adolescent intake of magnesium from fruit and vegetables (adjusted for FlavFV) and energy (residuals), and BMI-SD score in models with the predictor FlavFV (**a, c**) or sex, adult age at blood withdrawal, gestational age, birth weight, maternal gestational weight gain, and adolescent body surface area in models with the predictor uHA (**b, d**). Transformations of variables for analysis: reciprocal for HSI, $\log_e$ twice with constant = 1 for FLI, $\log_e$ for uHA, square root for FlavFV

for females. Thus, our findings provide novel evidence for a long-term early preventive relevance of flavonoids for type 2 diabetes already beginning in adolescence.

We are not aware of any other study which assessed the impact of flavonoids consumed during adolescence on any of the type 2 diabetes-related outcomes in adulthood investigated in this study. Therefore, our results can only be compared to studies investigating these exposures and outcomes either during adolescence or in adulthood.

Adolescent flavonoid intake and adult insulin sensitivity

Our observed prospective association between flavonoid intake during adolescence and insulin sensitivity in adulthood is consistent with evidence from observational studies conducted either in adolescence or adulthood. A meta-analysis of prospective studies with a follow-up of 9 to 28 years revealed an association between total flavonoid intake in adulthood and type 2 diabetes risk [1]. Prospective studies among adults published after this meta-analysis corroborated a lower type 2 diabetes risk among those with higher intakes of diverse flavonoid subclasses such as flavonols, flavanones and flavanols, estimated on either the dietary or the urinary biomarker level [26–28], albeit not on both levels as done in our study. Moreover, two cross-sectional studies among adolescents reported a lower odds of hyperinsulinemia for adolescents consuming fruit on a daily basis [29] and a higher insulin sensitivity in consumers of nutrient-rich vegetables compared to non-consumers [30]. Another cross-sectional study among adults found higher intakes of anthocyanins and flavones, but not total flavonoids or other subclasses, to be linked to lower fasting insulin levels and insulin resistance [31]. However, evidence from RCTs, all conducted among adults, is inconsistent: while fasting insulin and insulin sensitivity remained unaffected by interventions with polyphenol-rich juices of diverse fruits in the majority of RCTs [32–41], meta-analyses of RCTs revealed lowered fasting glucose and/or HbA1c concentrations following flavonol [42] or berry interventions [4]. Serum hippuric acid levels elevated after two berry interventions were correlated with fasting glucose levels and insulin secretion [43].

We can only speculate why the association of FlavFV-intake with fasting insulin and HOMA2-%S emerged solely among females: since females in our sample exhibited a lower insulin sensitivity than males, it is conceivable that flavonoids have a preventive effect primarily in more insulin resistant persons; alternatively, the sex-specificity

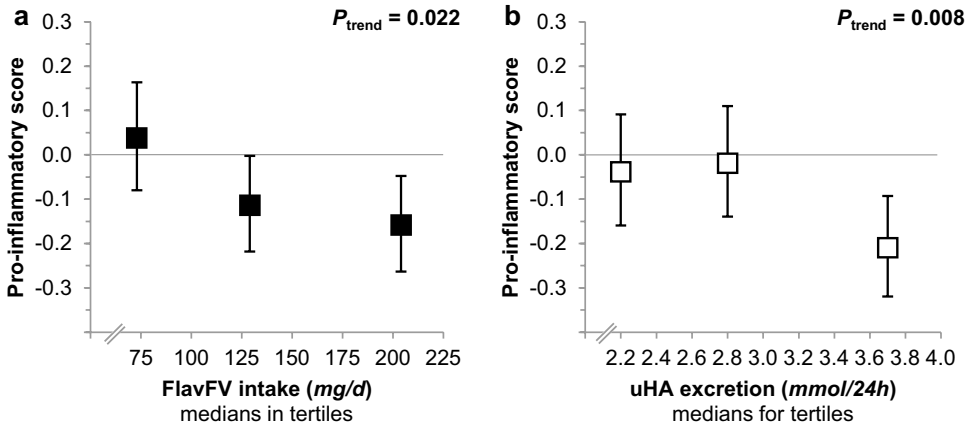

Fig. 2 Pro-inflammatory score (calculated from hsCRP, IL-6, IL-18, chemerin, leptin and adiponectin) in early adulthood by tertiles of dietary flavonoid intake from fruit and vegetables (FlavFV; **a**; $n = 265$) and urinary hippuric acid (uHA; **b**; $n = 238$) during adolescence. Data are geometric means and 95% CI adjusted for sex, adult age at blood withdrawal, maternal gestational weight gain, adolescent intake of saturated fat, vitamin C from fruit and vegetables (adjusted for FlavFV) and energy (residuals), and BMI-SD score in models with the predictor FlavFV (**a**) or sex, adult age at blood withdrawal, maternal gestational weight gain, and adolescent body surface area in models with the predictor uHA (**b**). Transformations of variables for analysis: $\log_e$ for the pro-inflammatory score (with constant = 2) and uHA, square root for FlavFV

may be attributable to higher relative intakes of FlavFV among females in our sample.

Adolescent flavonoid intake and adult hepatic steatosis

We are not aware of previous studies on validated indices of hepatic steatosis, namely HSI and FLI, in this context. The few available studies investigated the impact of flavonoid or fruit and vegetable intake primarily on liver enzyme activities [3, 32, 44], but these are less sensitive measures of hepatic fat accumulation than HSI and FLI. A prospective study observed an inverse association between fruit intake and GGT (γ-glutamyltransferase) concentrations in young US adults [44]. Furthermore, the only study measuring liver fat content by MRI among overweight Latino adolescents found that those with the highest intake of non-starchy vegetables had lower liver fat contents, however, using a cross-sectional study design [30]. Results from RCTs among adults were more controversial: While decreased ALT and GGT were reported in one RCT providing anthocyanins via a purple sweet potato drink for 8 weeks [3], another RCT providing the same amount via bayberry juice for 4 weeks observed no changes in ALT and AST [32], possibly due to the shorter study duration.

Adolescent flavonoid intake and adult chronic subclinical inflammation

Finally, the prospective long-term relevance of flavonoids consumed during adolescence for chronic subclinical inflammation in adulthood using single biomarkers or a pro-inflammatory score has not been assessed before. Observational evidence is only available from cross-sectional studies, which investigate the relevance of total flavonoids or their subclasses for inflammation scores or single inflammatory markers. Intake of flavonols or anthocyanidins, but not of total flavonoids, was associated with an inflammation score calculated from twelve individual inflammatory parameters among adults [45]. Of single inflammatory markers, hsCRP or IL-6 were most investigated. While no associations were found between any flavonoid and IL-6 among either adolescents or adults [46, 47], evidence regarding the relevance of total flavonoids, anthocyanins or flavonols for hsCRP is inconclusive among adults [31, 46, 48], and the only study conducted among adolescents found no relation [47]. Single studies addressing further inflammatory biomarkers suggested that total flavonoids, flavones or flavanones may be related to lower TNFα concentrations in adolescents [47], and lower IL-18 [46] or higher adiponectin [31] in adults. Similar inconsistencies are evident from RCTs: Two meta-analyses of RCTs on flavonoid or berry consumption in adults reported benefits for TNFα [4, 5], partially for IL-6 [5, 49], but not for hsCRP [4]. This picture is in part supported by other RCTs among adults using diverse fruit drink interventions (grapefruit, orange, grape, bayberry, blueberry or berry mix): a reduction of TNFα was shown in 2 out of 5 studies [32, 37, 41, 50, 51], no effect was found on hsCRP [32–34, 37, 41, 50–53], IL-6 [33, 37, 41, 50, 51] and adiponectin [41], while single studies demonstrated lowered leptin [40] or IL-8 [32] values.

Critical issues regarding flavonoid research

Taken together, this inconclusive evidence underlines two critical issues: Firstly, results from studies on fruit and vegetables may not be directly extrapolated to the action of flavonoids. Secondly, it is conceivable that the anti-inflammatory action of flavonoids does not primarily target concentrations of IL-6 and its downstream effector hsCRP, but rather impacts a broader inflammatory profile comprising additional pro- and anti-inflammatory parameters. The relevance for a broader inflammatory profile is indirectly supported by our results, where FlavFV and uHA are associated with the general pro-inflammatory score but show less pronounced effects on its individual inflammatory biomarkers. We cannot explain why we found an association with leptin only. While leptin has been ascribed pro-inflammatory and insulin-desensitizing properties [54], the other inflammatory markers are also consistently linked with insulin resistance and their responsiveness to flavonoids is supported by in vitro and animal studies [55, 56].

Total flavonoids and flavonoids from specific sources

Compared to total flavonoids or alternative sources of flavonoids (cocoa or nuts), adolescent intake of FlavFV was most consistently associated with adult risk markers of type 2 diabetes. Similar—albeit largely non-significant—directions in the associations between flavonoids from fruit, vegetables or juices (i.e. the components of our summary exposure FlavFV) support our hypothesis that for prevention during adolescence the entirety of flavonoids habitually consumed from fruit and vegetables is most relevant. Apples (with skin), red cabbage, strawberries, grapes and orange juice were the most important FlavFV sources in our sample, nearly contributing 50% of total FlavFV intake.

Potential mechanisms underlying the impact of flavonoids on T2DM Risk factors

Flavonoids act via multiple pathways to elicit metabolic, anti-inflammatory and anti-oxidant effects. One key mechanism improving hepatic steatosis, chronic subclinical inflammation and insulin resistance is the stimulation of PPARα and γ. Activation of these transcription factors mitigates inflammation by NF-κB inhibition, decreases steatosis by increased β-oxidation and further improves insulin sensitivity by favourably changing the balance between secretion of insulin sensitizing (e.g. adiponectin) and desensitizing cytokines (e.g. TNFα) [57].

Clinical relevance of habitual flavonoid intake during adolescence

The clinical relevance of our findings is particularly apparent for insulin sensitivity, which was 17% higher among females in the highest versus the lowest FlavFV tertiles providing 197 versus 74 mg FlavFV/d. The inter-tertile difference of 123 mg FlavFV/d can be achieved by one big unpeeled apple, 35 g blueberries, 58 g red cabbage or 2 oranges; with varying sources providing a wide diversity of flavonoids.

Strengths and limitations

Our study has several specific strengths. Firstly, the prospective design allows the investigation of long-term associations between adolescent flavonoid exposure and adult health outcomes. Secondly, we were able to estimate habitual flavonoid intake based on repeated exposure assessment from 3-d weighed dietary records and 24-h urine samples, two specifically detailed and accurate methods of exposure assessment. Thirdly, employing those two methods with their independent measurement error sources provides an insight into the causality of the observed associations, which would otherwise rely solely on one of the two methods with their intrinsic limitations (see below). Fourthly, we used a summary biomarker of FlavFV-intake, which we have recently validated specifically for healthy adolescents [13].

The main limitations of our study are the specific sources of measurement error intrinsic to both methods of FlavFV-intake estimation: While dietary records rely on self-reports and suffer from incomplete databases [11], 24-h uHA-excretion (especially the non-hydroxylated fraction) reflects not only flavonoids but also intake of other precursors like phenolic or benzoic acids (for details see our validation study [13]). Due to the wide application of benzoic acid as a food preservative, high consumption of certain preserved foods (e.g. fish, dairy & FV-products) would lead to some misclassification by the biomarker. As we could not adjust for non-flavonoid precursors, this may have resulted in a less precise association. Despite investigating possible confounding by diverse covariates—most importantly nutrients typically stemming from fruit and vegetables—we cannot fully preclude the possibility of residual confounding from unmeasured components of fruit and vegetables. The single measurement of outcomes in adulthood may be seen as a further limitation. Also, we used surrogate markers rather than hard endpoints such as type 2 diabetes incidence, which will only be discernible after a substantially longer follow-up. Nonetheless, higher levels of insulin resistance, hepatic steatosis and chronic subclinical inflammation can be expected to indicate a higher risk of later type 2 diabetes. Finally, our sample is non-representative due to its above-average

socioeconomic status, which may have led to selection bias and limit the generalizability of our results.

Conclusions

Taken together, our data suggest that a higher habitual flavonoid intake from fruit and vegetables during adolescence is relevant for the prevention of risk factors of type 2 diabetes in early adulthood. An increase in consumption by one to two portions of flavonoid-rich fruit and vegetables per day during adolescence may have considerable long-term benefits for insulin sensitivity, hepatic steatosis and chronic subclinical inflammation.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical standards The DONALD Study was approved by the Ethics Committee of the University of Bonn, Germany.

Informed consent All assessments in the DONALD Study were performed with parental written informed consent.

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