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The relevance of dietary insulin demand and dietary protein intake during adolescence for the development of body composition and the adult GH-IGF axis

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This thesis aimed to examine the relevance of dietary insulin demand and dietary protein intake during adolescence for the development of body composition and the adult GH-IGF axis. It resulted in the following:

Scientific papers

- **Joslowski G**, Goletzke J, Cheng G, Günther ALB, Bao J, Brand-Miller JC, Buyken AE. Prospective associations of dietary insulin index, glycemic index, and glycemic load during puberty with body composition in young adulthood. International Journal of Obesity (London) (2012) 36: 1463-1471. doi: 10.1038/ijo.2011.241

- **Joslowski G**, Remer T, Assmann KE, Krupp D, Cheng G, Garnett SP, Kroke A, Wudy SA, Günther ALB, Buyken AE. Prospective associations of different protein sources during childhood and adolescence with the growth hormone-insulin-like-growth-factor axis in younger adulthood. Journal of Nutrition (2013) 143(7):1147-54. doi: 10.3945/jn.113.175877

- Assmann KE, **Joslowski G**, Buyken AE, Cheng G, Remer T, Kroke A, Günther ALB. Prospective association of protein intake during puberty with body composition in young adulthood Obesity (2013) 21(12):E782-9. doi: 10.1002/oby.20516

- **Joslowski G**, Halim J, Goletzke J, Gow M, Ho M, Louie J C-Y, Buyken AE, Cowell CT, Garnett SP. Dietary glycemic load, insulin load, and weight loss in obese, insulin resistant adolescents: RESIST study. Clinical Nutrition (2014) published online: Jan 29. doi: 10.1016/j.clnu.2014.01.015. [Epub ahead of print]

Oral presentations

- **Joslowski G,** Goletzke J, Cheng G, Bao J, Brand-Miller JC, Buyken AE. Prospective associations between dietary insulin index during puberty and body composition in young adulthood.

  - International Journal of Obesity Supplements (2011) 1: S16

  - Proceedings of the German Nutrition Society (2011) 15: 10

- Buyken AE, **Joslowski G**, Goletzke J, Cheng G, Günther ALB, Bao J, Brand-Miller JC. Prospective associations between dietary insulin index, glycemic index and glycemic load during puberty and body composition in young adulthood. German Epidemiologic Society Meeting Abstract (2011) 6:64.

- Assmann K, **Joslowski G**, Buyken AE, Cheng G, Remer T, Kroke A, Günther AL. Prospective association of animal protein intake during puberty with body composition in young adulthood. German Epidemiologic Society Meeting Abstract (2012) 7:8.

- Assmann K, **Joslowski G**, Buyken AE, Cheng G, Remer T, Kroke A, Günther ALB. Prospective association of animal protein intake during puberty with body composition in young adulthood. Obesity Research & Clinical Practice (2012) 6:S1: 43 (I held this oral presentation on behalf of the study group)

- **Joslowski G**, Remer T, Assmann KE, Krupp D, Cheng G, Wudy SA, Günther ALB, Buyken AE. Prospective associations of dietary animal protein intake with the insulin-

like-growth-factor axis in adulthood. Proceedings of the Nutrition Society Australia (2012) 36: 47

Posters

- Buyken AE, **Joslowski G**, Goletzke J, Cheng G, Günther ALB, Bao J, Brand-Miller JC. Prospective associations between dietary insulin index, glycemic index and glycemic load during puberty and body composition in young adulthood. Diabetologia 2011; 54 (Suppl1) 542

- Buyken AE, Remer T, Assmann KE, Krupp D, Cheng G, Wudy S, Günther ALB, **Joslowski G**. Animal protein intake during puberty is related to the IGF-axis and insulin sensitivity in young adulthood. Diabetologia (2012) 55 (Suppl.1) S363

- **Joslowski G**, Halim J, Goletzke J, Dunkley M, Ho M, Louie JCY, Buyken AE, Baur L, Cowell CT, Garnett SP. Dietary glycaemic load, insulin load, and weight loss in obese and insulin resistant adolescents: RESIST study. Obesity Facts (Suppl.1) 224

Articles in national journals

- **Joslowski G**, Buyken AE. DONALD News: Insulin Index, glykämischer Index und glykämische Last der Ernährung in der Pubertät - Gibt es einen Einfluss auf die Körperzusammensetzung im jungen Erwachsenenalter?

    - Ernährungs Umschau (2012) 04: 189 (in German)

    - Pädiatrische Praxis (2012) 79: 65 (in German)

    - Ernährung im Fokus (2012) 12: 414 (in German)

SUMMARY

The relevance of dietary insulin demand and dietary protein intake during adolescence for the development of body composition and the adult GH-IGF axis

Obesity is associated with the development of chronic diseases such as type 2 diabetes. Obese children are likely to stay obese, resulting comorbidities may emerge already in childhood and tend to persist until adulthood. Puberty is a so-called critical period for overweight development, characterised by a physiological insulin resistance. Additionally, early life and the time around the adiposity rebound are potentially critical, developmental periods. The growth hormone insulin-like growth factor (GH-IGF) axis plays an important role during growth. It remains to be clarified whether it can be programmed by protein intake during growth. The **first aim** of this thesis was to examine the associations between dietary insulin demand during adolescence and its relevance for both adult body composition among healthy individuals and weight loss among obese adolescents with clinical features of insulin resistance. Under the **second aim**, prospective relations between dietary protein intake during different potentially critical, developmental periods and body composition as well as the GH-IGF axis in adulthood were investigated. Data came from two studies conducted in Germany and Australia; an ongoing, open cohort study, the DOrtmund Nutritional and Anthropometric Longitudinally Designed (DONALD) study and a randomised controlled trial, the Researching Effective Strategies to Improve Insulin Sensitivity in Children and Teenagers (RESIST) study, respectively.

Four analyses (studies I-IV) were conducted. **Study I** revealed that among 262 DONALD participants, a higher pubertal dietary insulin demand, but not a higher dietary glycaemic index or glycaemic load, was associated with higher body fat percentage in young adulthood. In **Study II**, a higher dietary insulin demand, estimated by dietary glycaemic load and insulin load, was related to less weight loss expressed as BMI as percentage of the 95th percentile (BMI %95 centile) among 91 RESIST participants. Inclusion of total energy intake in the model explained the observed associations between dietary insulin demand and change in BMI %95 centile. **Study III**, including 262 DONALD participants, indicated that a higher pubertal animal protein intake was independently associated with higher adult fat-free mass index (FFMI), but not fat mass index (FMI), in women. Among men, a higher pubertal animal protein intake was related to higher FFMI and lower FMI only after adjusting FFMI for FMI levels in young adulthood and vice versa. Plant protein intake was not associated with adult body composition among either sex. Higher animal protein intake around the adiposity rebound (n=220) tended to be related to higher adult FFMI among boys, but not girls. No relations were found between animal or plant protein intake in early life (n=159) and body composition in young adulthood. **Study IV**, also based on data from the DONALD study (n=213 and n=201, respectively), showed that a habitually higher animal protein intake during puberty was related to higher levels of adult IGF-I, IGFBP-3, and lower IGFBP-2, but not to IGFBP-1 among women. In turn, animal protein intake in early life (n=130) was inversely related to IGF-I levels in younger adulthood among males only. However, no association was observed between animal protein intake around adiposity rebound (n=179) and IGF-I in younger adulthood. No relations were observed between plant protein intakes in all three periods and adult GH-IGF axis.

In conclusion, results indicate that a lower dietary insulin demand and a higher dietary protein intake may be favourably related to adult body composition. Among women, a higher pubertal animal protein intake may induce an up-regulation of the GH-IGF axis which persists until adulthood. By contrast, inverse associations between higher animal protein intakes in early life and IGF-I concentrations among men support the idea that habitually higher animal protein intakes in this period may trigger an early programming of the GH-IGF axis. Although these findings need to be confirmed in other populations, a reduction of dietary insulin demand and a moderate increase in dietary protein intake may have beneficial effects in the prevention of obesity. Moreover, it needs to be identified which mechanisms lie behind observed associations between dietary animal protein intake and the GH-IGF axis so as to determine to what extent they reflect physiological adaptations or whether these associations indicate higher or lower risks of future diseases.

ZUSAMMENFASSUNG

Die Relevanz des ernährungsbedingten Insulinbedarfs und der Proteinaufnahme in der Jugend für die Entwicklung der Körperzusammensetzung und der GH-IGF Achse

Adipositas ist mit einer Reihe von chronischen Erkrankungen wie Typ 2 Diabetes assoziiert. Adipöse Kinder und Jugendliche haben ein hohes Risiko adipös zu bleiben und sind zudem anfälliger, bereits in jungen Jahren chronische Erkrankungen zu entwickeln, die bis ins Erwachsenenalter bestehen bleiben. Dabei ist die Pubertät durch eine vorübergehende physiologische Insulinresistenz gekennzeichnet und eine sogenannte kritsche Phase der Adipositasentwicklung. Zudem sind die frühe und mittlere Kindheit mögliche kritische Entwicklungsphasen. Die Wachstumshormon-*insulin-like growth factor* (GH-IGF) Achse spielt eine wichtige Rolle im Wachstum. Bisher ist unklar, ob die GH-IGF Achse durch die Proteinaufnahme programmiert werden kann. **Erstes Ziel** dieser Arbeit war Assoziationen zwischen dem ernährunsbedingten Insulinbedarf und der Körperzusammensetzung von gesunden Erwachsenen sowie der Gewichtsreduktion bei adipösen Jugendlichen mit Insulinresistenz und/oder Prädiabetes zu untersuchen. **Zweites Ziel** war, den prospektiven Zusammenhang zwischen der Proteinzufuhr in verschiedenen, potentiell kritischen Entwicklungsphasen und der Körperzusammensetzung sowie der GH-IGF Achse bei gesunden Erwachsenen zu untersuchen. Als Datengrundlage dienten sowohl die deutsche DOrtmund Nutritional and Anthropometric Longitudinally Designed (DONALD) Studie, eine offene, prospektive Kohortenstudie, als auch die australische Researching Effective Strategies to Improve Insulin Sensitivity in Children and Teenagers (RESIST) Studie, eine randomisierte, kontrollierte Interventionsstudie zur Gewichtsreduktion.

Vier Analysen (Studien I-IV) wurden durchgeführt. **Studie I** zeigte, dass bei 262 Probanden der DONALD Studie ein höherer Insulin Index und eine höhere Insulin Last, aber nicht ein höherer glykämischer Index bzw. eine höhere glykämische Last in der Pubertät mit einem höheren Körperfettanteil im jungen Erwachsenenalter assoziiert war. In **Studie II** war bei 91 Probanden der RESIST Studie ein höherer Insulinbedarf, geschätzt durch die insulinämische und glykämische Last der Ernährung, mit weniger Gewichtsverlust ausgedrückt als BMI in Prozent der 95. Perzentile (BMI %95 Perzentile) assoziiert. Die Berücksichtigung der Gesamtenergieaufnahme im Modell erklärte den Zusammenhang zwischen ernährungsbedingtem Insulinbedarf und Veränderung von BMI %95 Perzentile. **Studie III** schloss 262 DONALD Probanden ein und zeigte, dass eine höhere Aufnahme an tierischem Protein während der Pubertät bei Frauen unabhängig von anderen Covariaten mit einem höheren Fettfreie-Masse-Index (FFMI), aber nicht Fettmasse-Index (FMI) assoziiert war. Bei Männern hing eine höhere Aufnahme an tierischem Protein mit einem höheren FFMI und niedrigerem FMI nur nach Adjustierung des FFMI für FMI im jungen Erwachsenenalter und vice versa zusammen. Die Aufnahme von pflanzlichem Protein in der Pubertät war dagegen weder bei Frauen noch bei Männern mit der Körperzusammensetzung assoziiert. Allein bei Jungen war die Tendenz zu beobachten, dass eine höhere Aufnahme an tierischem Protein zum Zeitpunkt des *adiposity rebound* (n=220) mit einem höheren FFMI im jungen Erwachsenenalter einherging. Weder tierisches noch pflanzliches Protein in der frühen Kindheit (n=159) waren mit der Körperzusammensetzung im jungen Erwachsnenalter assoziiert. **Studie IV** basierte ebenso auf Daten der DONALD Studie (n=213 bzw. n=201) und zeigte, dass nur bei Frauen eine höhere Aufnahme an tierischem Protein während der Pubertät mit höheren IGF-I und IGF-Bindungsprotein(BP)-3, niedrigeren IGFBP-2, aber nicht IGFBP-1 Konzentrationen im frühen Erwachsenenalter zusammenhing. Die Aufnahme von tierischem Protein in der frühen Kindheit (n=130) war wiederum nur bei Männern invers mit IGF-I Konzentrationen im frühen Erwachsenenalter assoziiert. Zwischen der Aufnahme an tierischem Protein zum Zeitpunkt des *adiposity rebound* (n=179) und IGF-I Konzentrationen im frühen Erwachsenenalter konnten keine Zusammenhänge gefunden werden. Pflanzliches Protein war in keiner Phase mit der GH-IGF Achse im frühen Erwachsenenalter assoziiert.

Zusammenfassend legen die Resultate nahe, dass ein geringerer Insulinbedarf und eine höhere Aufnahme an tierischem Protein in der Pubertät vorteilhaft mit der Körperzusammensetzung im jungen Erwachsenenalter assoziiert sind. Bei Frauen bedingte ein höherer Verzehr an tierischem Protein in der Pubertät eine Hochregulation der GH-IGF Achse bis ins frühe Erwachsenenalter. Bei Männern hingegen deutet der inverse Zusammenhang zwischen einer höheren Aufnahme an tierischem Protein in der frühen Kindheit und niedrigeren IGF-I Spiegeln im frühen Erwachsenalter

auf eine langfrisige Programmierung der GH-IGF Achse hin. Obwohl diese Ergebnisse noch in anderen Populationen bestätigt werden müssen, könnten eine Reduktion des Insulinbedarfs und eine moderate Erhöhung der Proteinaufnahme positive Auswirkungen in der Adipositasprävention haben. Des Weiteren muss geklärt werden, welche Mechanismen hinter den beobachteten Zusammenhängen von tierischem Protein und der GH-IGF Achse liegen, um zu verstehen, ob es sich dabei um eine physiologische Adaptation oder ein erhöhtes/verringertes Risiko für spätere chronische Erkrankungen handelt.

TABLE OF CONTENTS

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

%95 centile	Percentage of the 95th percentile
%BF	Percentage body fat
%En	Percentage of total energy intake
95% CI	95% confidence interval
AUC	Area under the curve
ALSPAC	Avon Longitudinal Study of Parents and Children
ANCNPAS	Australian National Children's Nutrition and Physical Activity Survey
BIA	Bioelectrical impedance analysis
BMI	Body mass index
CDC	Centers for Disease Control and Prevention
CHOP	Childhood Obesity Programme
DEGS1	German Health Interview and Examination Survey for Adults
DEXA	Dual-energy X-ray absorptiometry
DIARY	German study which used the diabetes registry
DiOGenes	Diet, Obesity and Genes
DONALD	Dortmund Nutritional and Anthropometric Longitudinally Designed
EsKiMo	Ernährungsstudie als KiGGS Modul
FFQ	Food frequency questionnaire
FMI, FFMI	Fat mass index, fat-free mass index
FO	Fish oil supplement
GH, GHBP	Growth hormone, growth hormone binding protein
GI	Glycaemic index
GIP	Glucose-dependent insulinotropic peptide
GL	Glycaemic load
GLP	Glucagon-like peptide 1

ABBREVIATIONS

HbA$_{1c}$	Glycosylated haemoglobin
HEP	Healthy Eating Plan
HGI	High glycaemic index
HOMA	Homeostasis model assessment
HP	High protein
IASO	International Association for the Study of Obesity
IDF	International Diabetes Federation
IGF, IGFBP	Insulin-like growth factor, insulin-like growth factor binding protein
II	Insulin index
IL	Insulin load
IOTF	International Obesity Task Force
IQR	Interquartile range
IR	Insulin resistance
ISI	Whole body insulin sensitivity index
KiGGS	Examination Survey for Children and Adolescents
KORA	Kooperative Gesundheitsforschung in der Region Augsburg
LC	Low carbohydrate diet
LFD	Low fat diet
LGD	Low glycaemic load diet
LGI	Low glycaemic index
LP	Low protein
MRI	Magnetic resonance imaging
mTOR	Mammalian target of rapamycin
OA	Original article
OGTT	Oral glucose tolerance test
PC	Portion controlled diet

PREVIEW   PREVention of diabetes through lifestyle Intervention and population studies in Europe and around the World

RESIST   Researching Effective Strategies to Improve Insulin Sensitivity in Children and Teenagers

RFD   Reduced fat diet

RGL   Reduced glycaemic load diet

RQ   Research question

SD, SDS   Standard deviation, standard deviation score

SE   Standard error

SEM   Standard error of the mean

SOLAR   Study of Latino Adolescents at Risk for Diabetes

US   United States

WHO   World Health Organisation

1. INTRODUCTION

The obesity prevalence is rising since the 1980s, with a concomitant increase of affected children. Obese children are likely to stay obese and resulting comorbidities, like type 2 diabetes, hypertension or dyslipidaemia emerge already in childhood and are also likely to persist until adulthood. With regard to obesity development, there may exist critical periods in which stimuli like nutritional factors might have sustained effects on adult health. Particularly early life, adiposity rebound, and puberty are potentially critical and sensitive phases in which nutritional influences may affect an individual´s predisposition to later obesity [1, 2], hence providing potential for prevention. Puberty may be of specific relevance, since this phase is characterized by a physiological insulin resistance [3], changes in various hormone levels, including the insulin-like growth factor (IGF)-I, growth hormones as well as sex steroids [4], and concomitant changes in body composition.

The importance of diet composition for the prevention and management of obesity has been controversially debated and it has not yet been elucidated whether the conventional low fat diets are the best approach [5-7]. Official dietary guidelines in Germany and Europe still focus on restricting energy by reducing fat in the prevention of overweight as well as for weight loss [5, 8, 9]. A reduction of fat intake for short-term weight loss as well as long-term weight maintenance will lead to a relative increase in carbohydrate and/or protein intake compensating the reduction in fat. In this context, interest in the effect of dietary carbohydrate and protein intake on weight management has risen [10, 11]. With regard to high carbohydrate diets particular concern lies on excursions of postprandial glucose and insulin response, which might increase the risk of obesity and related chronic diseases [12, 13]. Hence, there exists increasing interest in the dietary glycaemic index (GI) estimating the relative glycaemic potency and glycaemic load (GL) indirectly estimating the dietary insulin demand of available carbohydrates consumed. However, high-GI foods influence both blood glucose and insulin levels and it has not yet been clarified which of these postprandial changes is potentially more relevant for an unfavourable development of body composition. Insulin secretion is also stimulated by dietary protein and moreover, dietary protein and fat may both act synergistically with carbohydrates, raising insulin levels and reducing postprandial glycaemia [14-16]. Therefore, the concept of the food insulin index (FII), estimating the dietary insulin demand may be of importance. With the knowledge of the FII and the foods energy content the insulin demand of a diet can be estimated. Of note, the FII

concept, unlike the GI concept, also considers foods with no or low amounts of carbohydrates [17].

High protein diets [18-20] as well as low-GI diets [21-23] have been reported to play a role in body weight regulation and modification of obesity related risk factors. The Diet, Obesity and Genes (DiOGenes) study, a multi-centre, randomized, dietary intervention investigated the efficacy of different low-fat diets, varying in protein content and GI, in preventing weight (re)gain and certain obesity-related risk factors in obese European families. A diet characterised by both a modest increase in protein content and a modest reduction in the GI led to maintenance of weight loss among adults [24]. Furthermore, the combination of a high protein and low-GI diet was found to be related to decreases in overweight or obesity rates among children and adolescents [25]. However, overall evidence relating insulin demand and protein intake during adolescence to body composition or weight loss is scarce and their relevance for prevention and management of obesity is not clear.

Protein intake particularly in childhood may not only be related to body composition but to the growth hormone (GH)-IGF axis because the GH-IGF axis plays an important role in foetal and childhood growth and metabolism [26]. Some, but not all [27, 28], intervention studies have shown a relation between higher milk intake and higher IGF-I [27, 29, 30] and IGFBP-3 [29], whereas prospective evidence suggest an inverse association between milk intakes in early childhood and IGF-I levels in later life [31, 32]. Prospective evidence covering different, potentially critical, developmental periods is lacking, so as to unravel whether such an inverse association between animal protein intake and the GH-IGF axis is confined to early life.

Therefore, the first aim of this thesis was to examine the dietary insulin demand and its relevance for both the development of body composition until young adulthood among a healthy population and weight loss among obese adolescents with clinical features of insulin resistance. The second aim was to examine dietary protein intake during different potentially critical, developmental periods and its relevance for the development of body composition and the GH-IGF axis in adulthood in a healthy population, respectively. Data came from two comprehensive studies conducted in Germany and Australia: an ongoing, open cohort study, the DOrtmund Nutritional and Anthropometric Longitudinally Designed (DONALD) study and a randomised controlled trial, the Researching Effective Strategies to Improve Insulin Sensitivity in Children and Teenagers (RESIST) study, respectively.

Outline of this thesis

This thesis begins with a **Theoretical background** (chapter 2), first describing the different predictors of postprandial insulin response which might play a role for the development of body composition and obesity. Thereafter, the epidemiology of obesity is described, with specific focus on Germany and Australia because the two studies on which this thesis is based were conducted in Germany and Australia. Consequences and determinants of obesity are summarized. This chapter also includes a summary of potential mechanisms of dietary protein intake as well as dietary glucose and insulin response and obesity. Consecutively, the regulation and the relevance of the GH-IGF axis for chronic disease risk are presented. In this context, the concept of critical periods for obesity development is outlined, focussing on early life, adiposity rebound and puberty as these periods are important in the context of this thesis. The last part of the theoretical background presents evidence from previous studies linking dietary GI and GL to body composition and weight loss and protein intake to body composition, obesity and the GH-IGF axis. Chapter 3 specifies the **Aims and research questions** addressed in this thesis and is followed by a short overview of the **General methodology** of the two studies on which the original articles of this thesis are based (chapter 4). Chapter 5 summarizes the results and includes the abstracts of the respective publications; copies of the original articles are included in the appendices (1-4). In the **General discussion**, the results of the original studies are discussed with regard to their scientific background (chapter 6). Finally, overall **Conclusions** are drawn and **perspectives** for future research are given (chapter 7). This thesis is cumulative and does not include detailed descriptions on the performed statistical analyses or the obtained results. The information on the analytical approaches, detailed presentations of the results and discussions of specific findings can be found in the original articles (appendices 1-4).

2. THEORETICAL BACKGROUND

2.1 Dietary predictors of insulin response

Studies among individuals with and without diabetes have found that higher levels of HbA_{1c} (glycosylated haemoglobin) were associated with higher risks of cardiovascular disease and all-cause mortality [33-35]. HbA_{1c} indicates the exposure to postprandial elevations of blood glucose among non-diabetic individuals. Therefore, the effect of food on glycaemic excursions might be relevant for disease risk including obesity. Likewise, postprandial rises of insulin levels might be of relevance for unfavourable health outcomes including weight gain and diabetes development. It has been assumed that insulin response is proportional to glucose response, hence supposing that glycaemic response is a precise predictor of insulin response, which is however not the case [36]. Postprandial insulinaemia has been shown to predict weight gain over a mean of 16.7 y, especially among individuals with high insulin sensitivity [37] as well as weight gain and change in waist circumference over 6 years in adults, especially among those consuming lower-fat diets [38]. Prospective long-term, observational studies found that glycaemic load, an indicator of insulin demand, was an independent risk factor for type 2 diabetes in women and men [39, 40]. Yet, the relevance of insulin response within disease risk including obesity has been controversially debated [41-43]. Mechanisms relating glucose response to the development of obesity have been established, whereas the relevance of insulin response for the development of obesity has not been fully elucidated. Potential mechanisms relating glucose and insulin response to body composition and obesity in order to disentangle effects of glucose and insulin response will be presented in chapter 2.2.1 (*Potential mechanisms relating glucose and insulin response to body composition and obesity*).

The concepts of dietary GI and FII originate from the idea to better estimate the dosage of insulin required to metabolise the postprandial glycaemic response in type 1 diabetes [44, 45]. In this context, the dietary GL as indirect and the insulin load (IL) as direct estimate of the postprandial insulin demand are of relevance. In addition, research has been interested in the intake of dietary protein, which has been shown to influence postprandial insulin response. The following chapters describe the determinants of insulin response firstly from the methodological and metabolic perspective of dietary GL and IL and secondly with regards to the macronutrient level (dietary protein and its sources).

2.1.1 Dietary glycaemic load

Carbohydrate containing foods are the major stimulus of postprandial blood glucose but their glycaemic potential varies with the type of carbohydrate [46]. It was assumed that the chemical structure of carbohydrates could be accounted for increases in blood glucose levels; "simple sugars" (mono- and disaccharides) being digested faster than "complex carbohydrates" (oligo- and polysaccharides) [47]. However, evidence suggested that these observations were not necessarily true as some starches resulted in similar blood glucose rises compared to glucose [48, 49]. This lead to the proposal of a new concept to classify carbohydrate containing foods based on their glycaemic potential – the concept of the GI [44].

Definition

The dietary GI is defined as the incremental area under the blood glucose response curve (AUC) following the intake of 50 g of available carbohydrates from a test food as compared with the AUC of blood glucose response induced by the same amount of carbohydrates ingested as glucose (reference food) [44]. GI measurements are done in at least 10 healthy individuals after an overnight fast. Capillary blood samples are taken during the first 2 hours after the ingestion of the test food to measure the glycaemic response, i.e. at baseline (0) and at 15, 30, 45, 60, 90 and 120 min after starting to eat the test meal [50]. The GI of a test food is calculated as the mean of individual ratios, i.e. (area under the 120 min glucose response curve elicited by the test food)/(area under the 120 min glucose response curve elicited by the reference food) multiplied by 100. The actual blood glucose response varies depending on the type of carbohydrates as well as a number of other factors such as the physical form of foods, the grade of food processing, the cooking method or the presence of organic acids (see also **Table 1**) [50].

Table 1 Factors affecting the glycaemic response to foods or meals (adapted from Brouns et al [50])

Food factor	Effect on glycaemic response / glycaemic index
Gross matrix structure	Higher when homogenised
Cell-wall and starch structure	Higher with ripening
Granular starch structure	Higher when gelatinised (e.g. through heat treatment)
Amylose and amylopectin content	Lower with higher amylose content, higher with increased amylopectin content
Gelling dietary fibre content	Reduced when gelling fibres are added
Organic acids, e.g. acetic acid	Reduced when acids are added
Amylase inhibitor	Reduced when added
Monosaccharide composition	Reduced with increased fructose content
Molecular composition of carbohydrate	Reduced with increased number of bonds other than $\alpha1-4$ and $\alpha1-6$
Resistant starch content	Indifferent when testing equal amounts of available carbohydrate

The GI is a qualitative measure of carbohydrate containing foods, classifying them according to their glycaemic response to a defined carbohydrate amount. Foods with a GI<55 are regarded as low-GI foods, whereas those with a GI>70 are considered high-GI foods [51]. However, the total amount of carbohydrates also affects the glycaemic response. Salmeron et al introduced the concept of the GL to describe the absolute glucose response induced by a serving of a carbohydrate-rich food [39]. The GL corresponds to the amount of available carbohydrates multiplied by their respective GI and can be interpreted as the amount of carbohydrates adjusted for its glycaemic potency.

Estimation

To estimate the GI of a diet, assignment of a published GI [52] to each carbohydrate containing food recorded in dietary records or 24h dietary recalls is required. Alternatively, when a food frequency questionnaire (FFQ) is used, mean dietary GI values for food groups need to be estimated and assigned. The total daily GL of a participant is obtained by summing the product of all consumed food's carbohydrate content (in grams) by the food's GI, divided by 100. The average dietary GI is estimated by dividing the total daily GL by the total daily carbohydrate intake multiplied by 100. While the dietary GI resembles the overall glycaemic potency of a diet, the dietary GL is an indirect estimate of the insulin demand.

2.1.2 Dietary insulin load

Analogous to glucose response, the actual insulin response varies depending on the type of carbohydrates as well as other factors such as the physical form of foods, grade of food processing or cooking method. Furthermore, insulin secretion is stimulated by dietary protein and dietary protein and fat may both act synergistically with carbohydrates, raising insulin

levels and reducing postprandial glycaemia [14, 15] (see also chapter 2.1.3). Beyond that, studies have shown that the postprandial insulin responses differed between several types of bread; for example, rye breads had lower insulin responses compared to white wheat bread [53, 54] and some rye varieties may be more insulin saving than others [55]. Several factors exist that mediate postprandial insulin response such as amino acids, glucose-dependent insulinotropic polypeptide (GIP) or glucagon-like peptide (GLP)-1 [56-58]. Since postprandial insulin responses are not always proportional to blood glucose responses, the postprandial insulin response cannot be estimated by GI, as already mentioned. Recent findings of an intervention trial among 10-13 healthy participants showed superiority of dietary GL over carbohydrate content alone to estimate postprandial insulinaemia [17], nonetheless, GL is only an indirect measure of postprandial insulin demand. Accordingly, a direct measure of the actual postprandial insulin response is needed.

Definition

The concept of the FII provides a classification of all foods according to their postprandial insulin response [59]. The FII is defined as the insulinaemic response (AUC) following the intake of 1 MJ of a food relative to the insulinaemic response to glucose i.e. the reference food (FII=100) [60]. Similar to GI, FII measurements are conducted in groups of 10 healthy individuals after an overnight fast. Capillary blood samples are taken during the first 2 hours after the ingestion of the test food to measure the insulinaemic response, i.e. at baseline (0) and at 15, 30, 45, 60, 90, 105 and 120 min after starting to eat the test meal [59]. The FII of a test food is calculated as the mean of individual ratios, i.e. (area under the 120 min insulin response curve elicited by the test food)/(area under the 120 min insulin response curve elicited by the reference food) multiplied by 100 [17, 59].

Estimation

Estimation of the dietary insulin index (II) requires the assignment of published FII values to each food recorded in dietary records or 24h dietary recalls. When using a FFQ, mean FII values for food groups need to be estimated and assigned if no tested FII value of food samples representative for FFQ items were available [61]. To date, 121 published FII values [17] and 6 recently measured values [62] are available. The total dietary IL of a participant is estimated by summing the product of FII, energy content and consumption frequency over all recorded food items. The average dietary II is calculated by dividing the total dietary IL by the total energy intake. Both, II and IL resemble the dietary insulin demand; however, they still have slightly different interpretations: While the dietary II is more a qualitative measure,

ranking foods according to their postprandial insulin response, the dietary IL gives a quantitative insight of the insulin demand of the diet.

2.1.3 Dietary protein

Although carbohydrate containing foods are the major stimulus of insulin secretion, they are not the only one. Already in the 1960s, researchers found that protein or amino acids given orally or intravenous stimulated insulin response in healthy or diabetic individuals [63-66]. Nonetheless protein rich foods vary in their insulin stimulating capacity [57, 59] and the relation might be different with respect to the protein source. Therefore, the following paragraphs focus on the macronutrient level of the diet and are presented by protein sources, i.e. dairy and meat products.

Dairy products

Several studies have examined the effect of dairy products such as milk, whey and cheese on postprandial glucose and insulin response and found that there exist differences in the metabolic responses.

Milk products have been shown to have a disproportionally high insulin response compared to what would have been expected from the corresponding glucose response among healthy participants [58, 67]. For example, the addition of **milk** to a low-GI spaghetti meal resulted in a 300% increase of the insulin response compared to the spaghetti alone, while there was no difference seen with regard to the glucose response. Thus, even the addition of a usual amount of milk (200mL) to a low-GI meal may elevate the postprandial insulinaemia to the extent of high-GI white bread alone [67]. In addition, milk products have been shown to reduce postprandial glycaemia, in fact induce hypoglycaemia in healthy participants [57, 58, 68], which may be explained by the high insulin concentrations.

On a food level, components like **lactose, whey and casein** are discussed to be responsible for high insulin responses. **Lactose** stimulates insulin response; however, it has been shown that consumption of milk products caused greater increases of insulin levels than the consumption of an equivalent amount of lactose among healthy adults. This suggests that there may exist milk components besides lactose which are responsible for increased insulin releases [58]. Studies which investigated the effect of dairy products with an equivalent amount of lactose demonstrated that **casein** produced similar insulin responses compared to milk and white wheat bread (reference food) among healthy participants. Conversely, **whey** had a more than 50% higher insulin response than white wheat bread and all dairy products

decreased the glucose response [57, 68]. However, not all studies confirmed an effect of whey on insulin response [69]. Earlier studies have investigated the influence of **cottage cheese**, which still contains **whey**, on insulin response, finding that it stimulated insulin response and decreased blood glucose in healthy and type 2 diabetic individuals [16, 70]. Furthermore, a **co-ingestion of cottage cheese and glucose** among type 2 diabetic individuals resulted in an increase of insulin response which was more than 2 times greater than that following the consumption of glucose alone, while no differences were observed with regards to glucose response between the two groups [16]. The sum of insulin responses of cottage cheese and glucose alone was smaller than the insulin response of the co-ingesting cottage cheese and glucose. Thus, studies in type 2 diabetic individuals indicate that the effect of protein and carbohydrates on insulin response may not be additive but synergistic. The reason for this is unknown [16]. Nonetheless, a synergistic effect of protein and carbohydrate would result in disproportional insulin responses which may have an implication on treatment of type 2 diabetes.

Overall, milk has insulinotropic effects; but this property might predominantly be related to the whey fraction.

Meat products

Earlier studies have examined the relation between meat products and postprandial glucose and insulin response. Similar to what has been observed for dairy protein, results showed that among healthy and type 2 diabetic individuals the ingestion of **meat protein** resulted in postprandial insulin increases, while the postprandial glucose response was rather decreasing [71, 72]. The **co-ingestion of meat protein and carbohydrates** given as glucose caused increases in postprandial glucose and a 2.7 to 4.5 fold higher increase in postprandial insulin response compared to meat protein alone. In addition, Nuttall et al observed that the co-ingestion of meat protein and glucose worked synergistically to increase the postprandial insulin response because it was 1.3 times greater than the sum of insulin responses of protein and glucose alone. Furthermore, the authors observed a dose-response relation between insulin response and the protein dose given (10 g to 50 g) [72]. In contrast, Krezowski et al observed that the co-ingestion of meat protein and glucose on postprandial insulin response was as high as the sum of insulin responses following glucose and protein alone suggesting an additive effect only. Observations for meat protein are similar to what has been observed for dairy products, however, the effect of meat protein seems to be smaller compared to dairy protein and whey protein in specific. Furthermore, it is controversial whether meat protein and

carbohydrate act synergistically. Understanding the relation of dietary protein intake and insulin secretion as well as potential mechanisms may identify novel targets for future diabetes therapies.

Potential mechanisms

The mechanisms for the insulinotropic effect of protein and dairy and/or meat protein in specific have not been fully elucidated. The amino acid profile and specific amino acids of the ingested protein itself may play a role in the hormonal response. Furthermore, the physical form of proteins, and/or bioactive peptides which are released during digestion seem to be involved.

Branched chain amino acids (leucine, isoleucine, valin) appear to have a higher insulinogenic effect than others, but also amino acids such as lysine, threonine, alanine or arginine have been found to stimulate postprandial insulin response [57, 73-77]. It is known that the mitochondrial metabolism is crucial for the coupling of amino acid and glucose recognition to the exocytosis of the insulin [78]. However, mechanisms by which amino acids enhance insulin secretion vary and depending on the amino acid different metabolic pathways are activated. Amino acids with a positive charge such as arginine increase insulin secretion by direct depolarization, while others like alanine which co-transport NA^+ can depolarise the cell membrane as a consequence of this co-transport [78]. Leucine may have allosteric effects on regulatory enzymes such as the glutamate dehydrogenase, hence increasing the glutamine-stimulated insulin secretion or stimulate insulin secretion via the activation of the mTOR (mammalian target of rapamycin) signalling pathway [79, 80], which will not be described in detail here. Overall, meat such as beef contains higher contents of amino acids compared to cow's milk or whey [81], but insulinotropic effects might be influenced by the physical form of proteins too [56]. Compared to solid proteins, liquid proteins pass the stomach faster and thus they are digested and absorbed quicker [82], resulting in higher plasma concentration of amino acids [57].

Another possible pathway may work through the activation of the incretin system, i.e. the system of insulinotropic hormones. Two relevant incretin hormones are the GIP and the GLP-1. They are released from the intestinal mucosa in response to food intake, enhancing insulin secretion in excess to what is caused by absorbed nutrients such as glucose or amino acids [83]. The GIP response may be a key factor for the higher insulin responses and subsequent decrease of glucose as seen after the ingestion of whey at least in healthy participants [57, 73]. In type 2 diabetic patients this insulinotropic effect of GIP is more uncertain because the

GLP-1 secretion and the insulinotropic activity of GIP are both impaired resulting in an impaired incretin effect [83]. In addition, carbohydrate and fat intake have been shown to mediate GIP response, whereas this effect is more uncertain for protein ingestion [84], although stimulation effects have been reported [73, 85, 86].

2.2 Body Composition and GH-IGF axis - Markers of later disease risk

Both body composition and the GH-IGF axis play a role in the development of chronic diseases. Obesity is related to comorbidities such as type 2 diabetes, hypertension or dyslipidaemia; the GH-IGF axis may play a role in the development of obesity, type 2 diabetes, and cardiovascular disease as well as different types of cancers. Knowledge on the development of body composition and the GH-IGF axis which may indicate an increased disease risk is needed. In this context, potentially critical developmental periods may play a role in the development of body composition and the GH-IGF axis.

2.2.1 Body composition and obesity

Definition of obesity

Overweight and obesity are defined as an abnormal or excessive body fat accumulation that may have detrimental health effects [87]. Among adults, body mass index (BMI) is the most commonly used, even though crude indicator for obesity, and it is estimated as weight / height^2 (kg/m^2). A BMI $\geq$25 kg/m^2 defines overweight, while a BMI $\geq$30 kg/m^2 classifies obesity [87]. BMI does not distinguish between weight associated with muscle or fat mass for which the BMI has been criticised [88-90] and the relation between BMI and body fat varies according to build and proportion [87]. To properly use and interpret BMI values it is important to also consider age, sex, ethnicity, physical activity, and body fat distribution.

During growth the BMI changes, which is why age- and sex-specific BMI percentiles are used to assess paediatric overweight and obesity [91]. These percentiles are typically based on national data and different cut-offs exist in different nations. In Germany there exist two datasets, the Kromeyer-Hausschild percentiles based on data from 17 regional studies conducted between 1985 and 1999 [92] and the newer KiGGS percentiles based on a representative Examination Survey for Children and Adolescents (KiGGS) conducted between 2003 and 2006 [93]. Because the KiGGS percentiles reflect the BMI distribution from 2003 to 2006, these data already include 50% more overweight children compared to the data assessed by Kromeyer-Hausschild (1985 to 1999) [94]. Hence, the use of the Kromeyer-

Hausschild percentiles to classify overweight and obesity is rational. A BMI above the 90th and 97th percentile of German reference curves is classified as overweight and obesity, respectively [92]. By contrast, in the United States (US) the 85th and 95th percentiles of the 2000 Centers for Disease Control and Prevention (CDC) growth charts are used to define overweight and obesity, respectively, and data was derived from 5 representative national surveys conducted from 1963 to 1994 [95-97]. To overcome the problem of different national definitions, the International Obesity Task Force (IOTF) has developed an international standard based on 6 nationally representative surveys from Brazil, Great Britain, Hong Kong, the Netherlands, Singapore and the US conducted between 1963 and 1993 to provides age- and sex-specific cut-offs that correspond to an adult BMI of 25 and 30 kg/m^2 at 18 years of age [98].

In research and evaluation of weight loss trials involving children and adolescents of different ages and sexes, BMI z-scores, also called standard deviation scores (SDS), are calculated using population based BMI reference data to adjust for changes which occur with normal growth. They provide a quantitative measure of how far away a child's BMI lies from the mean BMI value for sex and age, expressed in units of standard deviations (SD) [99]. However, change in BMI SDS can represent a broad range of weight changes, depending upon age, sex, initial BMI and reference data used [100-102]. Furthermore, reference data such as provided by the CDC were not designed to provide exact SDS values for children and adolescents beyond the 97th percentile, i.e. a BMI SDS >1.881 [103]. An alternative measure to assess and track the development of extremely heavy children and adolescents, their BMI can be described as a percentage of the 95th percentile (%95 centile) [103]. It has been shown that the association between weight change and change in BMI %95 centile is stronger than the association between weight change and BMI SDS in obese adolescents [104]. Using CDC data, a decrease in BMI SDS of ~0.25 was found to represent weight changes between -6.1 kg and +0.1 kg [104]. If a clinically significant change in BMI SDS was assumed to be 0.25 [101], the 15.1 year old boy who lost 9.6 kg (baseline BMI 39) would be classified as a treatment "failure" because the change in BMI SDS was ~0.16 (estimated from figure), whereas the 10.8 year old girl (baseline BMI 27), who maintained her weight would be classified as treatment "success" because the BMI SDS was ~0.25 (estimated from figure). Their respective decreases in BMI %95 centile were 13% and 4% [104], showing that BMI %95 centile may be a better indicator of weight change compared to change in BMI SDS among obese adolescents.

Besides BMI as a surrogate measure of body fatness researchers have suggested to also distinguish between fat mass and fat-free mass [90, 105-107]. Therefore, percentage body fat (%BF) as well as fat mass index (FMI) and fat-free mass index (FFMI), calculated as ([weight · %BF] / height^2) and ([weight - weight · %BF] / height^2), respectively, are frequently used. To define excess of body fat, %BF reference values for adults [108, 109] as well as for children and adolescents [110] have been published. For children and adolescents, reference percentile curves were developed using total body fat data of 1,985 British Caucasian children aged 5-18 years measured by bioelectrical impedance analysis (BIA). %BF above the 85th and 95th percentile is defined as overfat and obese, respectively [110].

For this thesis Kromeyer-Hausschild reference curves were used to derive sex- and age-independent SDS for the analyses of the DONALD study, since KiGGS percentiles have not yet been available for the first analysis. CDC growth charts were used for estimations within data of the RESIST study. Within the RESIST study weight loss was assessed using BMI %95 centile to better describe changes among obese participants. Overweight and obesity were defined according to the International Obesity Task Force criteria to allow comparability.

Epidemiology

Worldwide obesity has nearly doubled since 1980 and became a major health burden [111]. The International Association for the Study of Obesity (IASO) and the IOTF analysis estimated that in 2010 approximately 1.5 billion adults were overweight, including 475 million who were obese. Furthermore, up to 200 million school aged children were classified as overweight with 40-50 million of them being obese [112].

For Germany, the German Health Interview and Examination Survey for Adults (DEGS1) conducted from 2008 to 2011 and KiGGS conducted from 2003 to 2006 provide the latest representative data. According to DEGS1, 67.1% of men and 53.0% of women (≥18 years of age) were overweight and 23.3% of men and 23.9% of women were obese, with an evidently higher prevalence in the older age groups [113]. Using the definition for overweight and obesity by Kromeyer-Hausschild as described above [92], the KiGGS survey revealed that 15.4% of children between the ages of 7 and 10 were overweight (BMI > 90th percentile) and 6.4% were obese (BMI > 97th percentile). Among adolescents aged 11-13 years, 18.6% were overweight and 7.2% were obese. Among 14-17 year olds, less adolescents were overweight and more obese, i.e. 17.1% and 8.5%, respectively [94]. Similar proportions were observed among girls and boys.

For Australia, the 2011 to 2012 National Health Survey provides data on overweight and obesity prevalence, revealing that 63.4% adults (≥18 years of age) were overweight and 28.3% were obese. More men (70.3%) were overweight or obese compared to women (56.2%). Rates for both men and women have increased since 2007 to 2008 (67.7% for men and 54.7% for women) [114, 115]. Similar to what has been observed in Germany, the highest rate of overweight and obesity were observed in the older age groups [115]. Among children and adolescents aged 5 to 17 years, 25.7% were overweight and 7.6% obese (defined according to IOTF [98]). The proportion of girls who were overweight or obese was higher than for boys (27.1% compared to 23.6%) [116]. There has been no change in the proportion of children who were overweight or obese between 2007 to 2008 and 2011 to 2012. The newest data specifically on adolescents is derived from the 2007 Australian National Children's Nutrition and Physical Activity Survey (ANCNPAS) which used the IOTF cut-offs. Among 9-13 year olds, 25% boys were overweight and 7% were obese. Girls had a higher overweight prevalence of 30% compared to boys, but similarly 7% of girls were obese. The overweight prevalence of older adolescents (14-16 years of age) was 25% for boys and 23% for girls; the obesity prevalence was 6% for boys and 7% for girls [117]. To date, prevalence data on overweight and obesity among specific ethnic groups, including Aboriginal and Torres Strait Islander children and adolescents, is missing. Though, it has been recognised that overweight and obesity is a problem among these ethnic groups, even though less well understood [118]. However, ANCNPAS was not designed to collect information on representative samples of children living in remote areas or on those children of indigenous origin. Hence, only 3% of children included in the survey were of indigenous origin [117].

Consequences

Consequences of obesity are diverse. Obesity is a major risk factor for multiple health problems, including several of the major causes of death and disability, and psychosocial problems; affecting individual's health and quality of life along with the whole society due to economic consequences.

Adverse health effects associated with an increased BMI and in fact often overweight, not only obesity, are sleep apnoea, reproductive disorders, osteoarthritis, gall bladder disease, liver disease such as non-alcoholic fatty liver, hypertension, type 2 diabetes, and cancer [119]. Furthermore, childhood obesity is also associated with metabolic and cardiovascular complications such as impaired glucose regulation, type 2 diabetes, hypertension, dyslipidaemia, and systemic "low grade" inflammation [120]. These complications develop

already during childhood and are closely linked to concomitant insulin resistance/ hyperinsulinaemia [121] and degree of obesity [122]. Affected children are likely to stay obese and existing comorbidities are likely to persist until adulthood.

Obesity is strongly associated with insulin resistance, which is why obesity, particularly central obesity, is an important risk factor for type 2 diabetes [121]. A recent review reported consistent evidence that overweight in childhood and adolescence was associated with increased risk of type 2 diabetes in adulthood [123]. Type 2 diabetes was long considered to be an adult disease [124]. For adults, the incidence of type 2 diabetes has risen since the 1970ies. In 1985, the best prevalence data available suggested that 30 million people worldwide had diabetes [125]. According to the International Diabetes Federation (IDF), the prevalence increased by almost 13 times with 382 million adults aged 20-79 years having diabetes in 2013, approximately half of them undiagnosed [126]. The prognosis for 2035 is that this number will increase to 592 million people [126]. Among children and adolescents, the prevalence and/or incidence of type 2 diabetes vary substantially between countries, age categories and ethnic groups which is due to variations in population characteristics and methodological dissimilarities between studies [127]. There exist only few population based studies. In 2001, the SEARCH for Diabetes in Youth Study in the US identified that the proportion of type 2 diabetes ranged from 19/100,000 (non-Hispanic white youth) to 174/100,000 children and adolescents (American Indian youth) aged 10-19 years [128]. The overall prevalence of type 2 diabetes was 42/100,000 children and adolescents. Newer data from 2002-2005 indicated a prevalence of 18/100,000 children and adolescents for 10-19 year old non-Hispanic whites [129] which was similar to what had been observed earlier for this ethnic group [128]. The 2002-2005 incidence of type 2 diabetes among 10-19 year olds ranged from was 3.7/100,000 (non-Hispanic white youth) to 27.7/100,000 person years (Navajo youth) [129, 130]. A German cross-sectional survey among 721 school-leaving students with a mean age of 15.5 years found that 2.5% of adolescents had an impaired fasting glucose, impaired glucose tolerance or type 2 diabetes [131]. Another German study which used the diabetes registry (DIARY) found a type 2 diabetes prevalence of 2.3/100,000 children and adolescents aged 0-20 years in Baden-Württemberg [132], hence much lower compared to the US prevalence. To date, there are no type 2 diabetes incidence data available German children and adolescents. For Australia, accurate data on the type 2 diabetes prevalence of children and adolescents are not yet available. However, two relatively recent studies using data from a review of prospectively recorded diabetes data (Western Australia Childhood Diabetes Database) [133] and from a prospective population-based incidence study

(Australian Paediatric Endocrine Group New South Wales Diabetes Register) [134] both showed an incidence of type 2 diabetes among 0-19 year olds of 2.5/100,000 person-years among Indigenous children and adolescents. Non-Indigenous children and adolescents had an incidence of 0.2/100,000 person-years. These incidences are also lower than what has been observed in the US. Differences in the incidence between Indigenous and non-Indigenous children and adolescents which were observed in the US were also seen in Australia, but at a lower level. Reasons for observed differences are not entirely clear. However, different levels of physical activity, dietary habits and genetics may have led to different increases of obesity and type 2 diabetes over time. The fact that the diabetes prevalence is currently rising leads to increases in complications because an early onset of type 2 diabetes is associated with increased risk of morbidity and mortality [135, 136].

Besides the relation of obesity and type 2 diabetes, obesity is also considered a major risk factor for cardiovascular diseases, the world's leading cause of death [137]. This risk is increased due to rises in blood pressure and lipids, i.e. hypertension and dyslipidaemia. In adults, positive associations between obesity and hypertension [138-140] as well as dyslipidaemia [139] have been shown. Similarly unfavourable associations have been observed in children and adolescents [123, 141-143]. Although there are studies showing an association between childhood BMI and cardiovascular outcomes, evidence is lacking showing effects independent of adult BMI [123]. Moreover, obesity has also been related to cancer risk and it is proposed that the GH-IGF axis plays a key role within the development and progression of cancer [144] (see chapter 2.2.2 for details).

In addition to physical consequences there also exist psychosocial ones. Overweight and obesity are associated with stigmatization and discrimination. This contributes to difficulties and inequities in working life, increased vulnerability to low self-esteem, poor body image, and risk of developing serious psychological problems such as depression or anxiety disorders. Coping strategies to deal with the psychological pressure include dysfunctional eating behaviour and exercise avoidance [145, 146]. Among the overweight and obese youth, psychological consequences gain more recognition [147]. It has been found that weight-related teasing was consistently associated with body dissatisfaction, low self-esteem, high depressive symptoms, and thought and or attempt of suicide and nearly doubled the rates of all psychological complications in a sample of 4,746 adolescents [148].

Alongside with the described health consequences of obesity, there exists an economic burden for the whole society. According to the World Health Organisation (WHO) in 2000, the

worldwide economic costs of obesity in developed countries ranged from 2% to 7% of the total health care costs [87]. In 2008, the overall German costs for obesity (defined according to ICD 10 (E65-E68), i.e. BMI $\geq$30 kg/m^2) among all ages were 863 million Euro [149], accounting for 0.3% of the German total ICD 10 disease costs and hence much lower than the proportions of costs estimated by the WHO. It is of note, that the German total costs for obesity were 70, 65, and 728 million Euro for children and adolescents (<15 years of age), younger adults (15 to 30 years of age), and adults (>30 years of age), respectively [149]. Furthermore, results from the KORA (Kooperative Gesundheitsforschung in der Region Augsburg) survey found that medical costs were about three times higher for individuals with severe obesity (BMI $\geq$35 kg/m^2) compared to those with normal weight [150]. An Australian study analysing 5-year follow-up data from the Australian Diabetes, Obesity and Lifestyle study which started in 1999-2000 showed that among adults, 30 years or older, the health costs related to overweight (BMI $\geq$25 kg/m^2) accounted for 18.8 billion Australian Dollar [151], i.e. 12.5 billion Euro [152]. The costs for obesity (BMI $\geq$30 kg/m^2) were 8.3 billion Australian Dollar [151], i.e. 5.5 billion Euro [152]. Overall, appropriate treatment would have enormous benefit, both to patients in terms of increasing life expectancy and quality of life, as well as in economic terms for the society and the health-care system.

Determinants

Determinants of obesity are manifold. There is general agreement that the obesity epidemic cannot be solely attributed to a genetic change since it is taking place in stable populations over a relatively short period of time [153]. However, some hereditary factors can influence an individual's susceptibility to become overweight or obese. There exist rare genetic defects (e.g. mutations of gene encoding leptin or the leptin receptor) which result in severe obesity [154] and furthermore, gene defects which have been found to impair satiety and affect appetite control [155]. Overall, studies in families, adoptees, twins and also adopted twins have shown that heritable factors are likely to be responsible for 40-75% of inter-individual variation in BMI [154, 156]. Besides hereditary factors, any factor increasing energy intake or decreasing energy expenditure will lead to obesity in the long-term [153, 157]. The expression "obesogenic environment" has been coined [158], which is characterised by a lifestyle lacking of physical activity and present inactivity, the availability of energy-dense foods and drinks, their promotion in the media, large portion sizes and changes in the home and working environment [153, 157].

Among adults many dietary weight loss strategies have been focusing on the macronutrient content of the diet, i.e. low-fat diets, low-carbohydrate diets or moderate-protein-content (30%) diets. They have been shown to have beneficial short-term effects on obesity while long-term effects still need to be determined. It is noteworthy, that (long-term) compliance was shown to be positively affected by moderate-protein-content diets [159]. A recent meta-analysis emphasised that modest instead of large increases in protein content are probably more likely to have favourable effects on body weight or BMI [18]. In turn, little is known about the optimal dietary approach for weight loss and weight maintenance in obese children and adolescents. To date, studies conducted among overweight or obese children and adolescents to determine whether altering the macronutrient distribution of the diet has any impact on weight loss report inconclusive results [160]. One focus of this thesis was the relevance of dietary protein intake for body composition and obesity among children and adolescents; chapter 2.3.2 will present an overview of existing literature. Mechanisms relating dietary protein intake to body composition will be described in the following paragraphs in this chapter. Furthermore, the quality of carbohydrates, including the concept of the dietary GI, has received considerable attention in relation to body composition and obesity [161, 162]. Low-GI and/or GL diets have been reported to play a role in body weight regulation and modification of obesity related risk factors [21-23]. Chapter 2.3.1 will give an overview of the existing literature linking dietary GI and GL to body composition and obesity among children and adolescents. Potential mechanisms linking glucose response to body composition and obesity have been established, but to date, the relevance of insulin response for the development of obesity has not been fully elucidated. Studies examining the relation of the dietary insulin demand, as estimated by the dietary II and IL, with body composition are lacking thus far. Discussed mechanisms for glucose perhaps apply for insulin response too or that insulin response might have its own importance within the development of obesity. Hence, potential mechanisms relating glucose and insulin response to body composition and obesity will be presented in the last part of this sub-chapter, in order to disentangle effects of glucose and insulin response.

Potential mechanisms relating dietary protein and body composition

Potential mechanisms linking high protein intake with optimal body weight regulation are related to increased satiety, increased thermogenesis, lower energy efficiency, and maintenance of fat-free mass. They will be briefly described in the following paragraphs, focussing on a high protein diet rather than acute effects of high protein consumption. In addition, the potential effect of higher protein intake on muscle mass will be described.

Among macronutrients, dietary protein is the most satiating, followed by carbohydrates and fat [163, 164]. Mechanisms linking dietary protein intake and satiety include an increased fat oxidation, which also itself has been suggested to reduce appetite [165] as well as an increased production of ketone bodies from ketogenic amino acids (e.g. leucine, lysine) [164]. Furthermore, the digestion rate may play a role: Rapid gastric emptying and a postprandial increase in plasma amino acid concentrations after ingestion of specific proteins (e.g. whey vs. casein) [69] may increase satiety because of a greater stimulatory effect on gastrointestinal hormones such as cholecystokinin and GLP-1, but sufficient evidence is still lacking [164, 166].

More importantly, only satiety induced by high protein diets is primarily related to elevated energy expenditure [163, 166]. The daily energy expenditure consists of (i) basal metabolic rate, which entails sleeping metabolic rate and energy cost of arousal, (ii) diet-induced energy expenditure, and (iii) activity-induced energy expenditure [20, 163]. Dietary protein intake primarily affects the diet-induced energy expenditure. In fact, dietary protein has the highest thermic effect (20-30%), followed by dietary carbohydrates (5-10%) and dietary fat (0-3%) [167]. Adenosine-triphosphate is required for the initial steps of metabolism, storage and oxidation and may therefore mediate the short-term effect on diet-induced energy expenditure. The metabolic efficacy of dietary protein oxidation is dependent on amino acids, but overall it is relatively low compared to glucose or fatty acids [168]. This lower energy-efficiency of dietary protein contributes to higher diet-induced energy expenditure of a high protein diet, which in turn may be related to higher satiety feeling [164]. In addition, it has been shown that the consumption of pork meat resulted in a 2% higher 24h energy expenditure, including an increase in sleeping metabolic rate and diet-induced energy expenditure, compared to the ingestion of soy [169]. Thus, effects on energy expenditure may vary due to different protein sources (also depending on the level of energy intake in relation to energy requirement) [163], which may be a result of an increase stimulation of protein synthesis and protein turnover [164]. Since protein synthesis requires an adequate availability of essential amino acids, the intake of animal protein, which generally has a higher essential amino acid content compared to dietary plant protein [170], may result in more protein synthesis and hence larger increases of energy expenditure compared to plant protein intake [164].

A higher protein intake may also support mechanisms of sparing fat-free mass by its metabolic inefficiency and its ability to increased energy expenditure [163, 164, 171]. In fact, fat-free mass is the major determinant for basal energy expenditure. Not only the percentage

of energy, but the absolute protein intake, has to be considered. Within an energy restricted diet, the required daily protein intake needs to be within the range of 0.8g/kg and 1.2g/kg body weight to maintain the original absolute protein intake and to only limit carbohydrate and fat intake. Within this range, a daily protein intake of 0.8g/kg body weight is sufficient for substantial weight loss, subsequent weight maintenance, and a decrease in %BF, whereas 1.2 g/kg body weight is necessary for improvement of fat-free mass and a sustained resting energy expenditure [164, 171].

The above described mechanisms focus on the relation between higher dietary protein, weight loss and weight maintenance. On the other hand, dietary (animal) protein intake may be directly related to increases in muscle mass. An anabolic effect of essential amino acids on muscle mass was observed in small experimental studies among younger and older adults [172]. Especially branched chain amino acids, in specific leucine, may be involved. The potential biochemical pathway by which leucine may stimulate the muscle protein synthesis may work through the activation of the protein kinase mammalian target of rapamycin (mTOR) and its downstream effectors eukaryotic initiation factor 4E (EIF4E) and ribosomal S-6 kinase (S6K1) [173]. Furthermore, it has been suggested that branched chain amino acids, and again leucine, may inhibit proteolysis in skeletal muscles [174, 175]. Therefore, dietary animal protein intake may be more relevant in these anabolic processes due to the higher content of essential amino acids than dietary plant protein [170].

Potential mechanisms relating glucose and insulin response to body composition and obesity

The majority of short-term studies found that low-GI meals were followed by an increased satiety, decreased hunger or lower voluntary energy intake compared to high-GI meals [176]. Mechanisms linking the consumption of high-GI diets to body composition include reduced satiety signalling, as fully gelatinized starches in high-GI foods do not reach the lower parts of the ileum and hence do not stimulate satiety signals such as GLP-1 or cholecystokinin [176-179]. Nonetheless, satiety may not be exclusively related to postprandial glucose response as described by the dietary GI. With regards to insulin response, Graaf et al suggested that insulin may not be the best biomarker of satiety, since it is influenced by metabolic processes involving glucose and incretin responses [178]. However, a lower insulin response has been related to improved satiety in some [54, 55, 180-182], but not all studies [183]. Overall, satiety is possibly affected by the type of carbohydrates and their absorption rather than insulin concentrations per se [54].

The consumption of a high-GI meal results in a rapid and high postprandial increase of blood glucose levels. This relative hyperglycaemia is accompanied by elevated concentrations of GLP-1 and GIP, which potently stimulate insulin secretion and inhibit glucagon secretion in the early postprandial phase. The high insulin-glucagon ratio results in increased anabolic effects: stimulating the uptake of glucose and fatty acids into insulin sensitive tissues (muscle, adipose tissue, and liver), stimulating carbohydrate oxidation, and suppressing fat oxidation [177, 184]. Over the long term, this may result in a preferential direction of nutrients away from oxidation in the muscle towards storage in fat [176] and suppression of lipolysis [161]. Nutrient absorption declines in the middle postprandial phase, while high insulin and low glucagon levels persist. Subsequently, glucose levels fall rapidly and often below baseline levels, a condition which is named "reactive hypoglycaemia". In addition, circulating fatty acids, the other metabolic fuel, are reduced. These low concentrations of metabolic fuels lead to enhanced levels of counter-regulatory hormones which in turn stimulate hunger and food intake to restore energy homeostasis, and also increase free fatty acid concentrations in the late postprandial phase [13, 161, 177]. Over the long-term, recurrent metabolic responses to high-GI diets will gradually increase food intake and together with even small energy imbalances promote weight gain and obesity [13, 23]. Moreover, counter-regulatory hormone responses following a high-GI meal may have proteolytic effects and increase the loss of lean body mass over time. This in turn may reduce resting energy expenditure [177, 185] and eventually lead to a gain in body weight. In general, high-GI diets result in a sequel of postprandial glycaemia, insulinaemia, counter-regulatory hormones, and increases in free fatty acids [13]. Healthy and active individuals may adjust to these metabolic challenges by increasing the insulin sensitivity of their peripheral tissues [186], but less insulin sensitive individuals must increase their insulin secretion in order to re-establish glucose homeostasis [187]. Higher insulin levels are required to compensate relative hyperglycaemia induced by high-GI diets, which subsequently contribute to the development of insulin resistance [188]. Insulin resistance leads to compensatory increased hyperinsulinaemia and an increased the demand on β-cells [13]. Furthermore, higher levels of counter-regulatory hormones and free fatty acid concentrations in the late postprandial phase (as described above) can contribute to insulin resistance too [13]. The habitual consumption of diets with a high dietary GI may initiate a cycle of hyperinsulinaemia, reduced insulin sensitivity, and insulin resistance and may thus contribute to an increased risk of obesity as well as type 2 diabetes over the longer-term [13, 189, 190].

These mechanisms described for high-GI meals are related to both postprandial glucose and insulin responses. Foods with a high dietary II specifically stimulate postprandial insulin, but not necessarily glucose response. Hence some of the mechanisms may primarily apply for insulin responses in relation to body composition and obesity, including the redirection of nutrients towards storage in fat [176], suppression of lipolysis [161], and reduction of insulin sensitivity [13]. In addition, cross-stimulation of both insulin and IGF-I secretion may promote development of obesity [191]. In vitro as well as in vivo studies have shown that IGF-I plays an important role during adipogenesis [192, 193]. IGF-I was found to exert distinct effects on stem cells to stimulate proliferation and differentiation of pre-adipocytes, which may therefore contribute to body fat formation. Additionally, IGF-I may stimulate cellular glucose uptake in pre-adipocytes and adipocytes as well as it may increase lipogenesis and inhibit lipolysis in adipocytes [192-194]. On the other hand, the GI of a meal has been shown to acutely affect the GH-IGF axis, i.e. a high-GI meal decreased IGFBP-3 levels suggesting an increased available amount of free biologically active IGF-I in the tissue as it is no longer bound to IGFBP-3 and the acid labile subunit (see also chapter 2.2.2) [195].

Taken together, postprandial glucose and insulin response are interconnected and both seem to be related to body composition development. It is however not clear whether glucose or insulin response is more important for the development of body composition and obesity.

2.2.2 GH-IGF axis

The endocrine system of the GH-IGF axis plays an important role in postnatal growth and development, which are important determinants for the final phenotype of the adult organism [196]. This system includes three endocrine organs, hypothalamus, pituitary, and liver, consecutively releasing hormones to regulate anabolic processes [197].

Regulation of the GH-IGF axis

The structure of the GH-IGF axis is illustrated in **Figure 1**. GH-releasing hormone (GHRH) and a small amount of ghrelin are released by the hypothalamus; most of circulating ghrelin is, however, secreted by the stomach [198]. They both bind to their respective receptors in the pituitary to stimulate GH secretion. The hypothalamic factor somatostatin on the other hand inhibits GH secretion [197]. Together with GHRH, the oscillatory release of somatostatin may be responsible for the pulsatile secretion of GH [199]. Through binding to GH-binding protein (GHBP) in the circulation, GH bioactivity and its cell receptor binding is modulated and partly limited [200]. GH acts through specific cell-surface receptors stimulating the release of

IGF-I primarily from the liver and other tissues such as skeletal muscle cells [201-203]. IGF-I mediates most of the anabolic actions of GH including induction of cell growth, prevention of apoptosis, and induction of cellular differentiation [26]. Signalling works through the IGF-I receptor. IGF-I can bind to receptors that are present either on the cell of its own origin and stimulate growth (autocrine) or by binding to receptors on adjacent cells such as epithelial cell types that do not synthesise IGF-I but are stimulated to grow by locally secreted IGF-I (paracrine) [197, 204]. IGF-I also acts through endocrine mechanisms. In the circulation as well as extracellular, IGF-I is bound to IGF-binding proteins (IGFBP) that coordinate and regulate its biological functions. IGF-I circulates almost entirely (>99%) bound to IGFBPs [197, 205]. Through a negative feedback mechanism IGF-I inhibits GHRH and GH secretion [197].
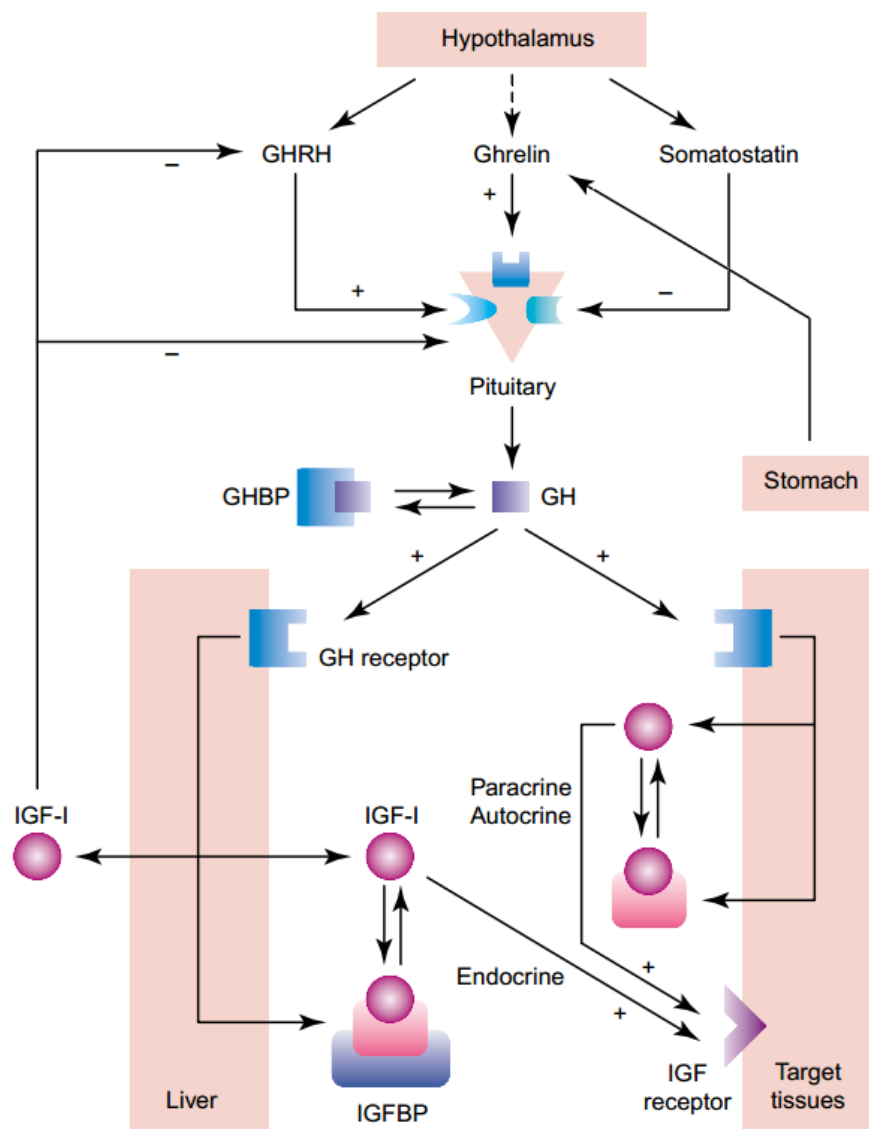
Figure 1 Structure of the GH-IGF axis (after Holt [197]). GH, growth hormone; GHBP, GH-binding protein; GHRH, GH-releasing hormone; IGF, insulin-like growth factor; IGFBP, IGF-binding protein.

Nutrition is a major regulator of circulating **IGF-I**. Scarcity of energy and/or protein results in decreased serum IGF-I concentrations [206]. After fasting, an optimal intake of both energy and protein is required to restore IGF-I levels in the circulation. However, while even a low protein intake is able to increase IGF-I in the presence of adequate energy intake, this effect is not achieved when both protein and energy intake are low [207]. Hence, there seems to be a threshold of energy requirement below which protein fails to increase IGF-I after fasting. Furthermore, when energy is restricted, an adequate carbohydrate, not fat, content of the diet is an important determinant of the responsiveness of IGF-I to GH [206]. With regards to protein intake, it has been shown that essential amino acids are more potent to increase IGF-I than non-essential amino acids after fasting [208]. Therefore, essential amino acids within the diet are necessary for optimal restoration of IGF-I levels, when protein intake is low [206]. Nevertheless, also non-essential amino acids (such as glutamine [209] or arginine [210]) or the combination of amino acids (lysine and arginine [211]) increase GH concentrations and lead to the stimulation of hepatic IGF-I secretion, even though this might be to a lower extent. As it is important for this thesis, the relevance of dietary protein intake for the GH-IGF axis will be presented more detailed in chapter 2.3.3.

There is a variation of IGF-I levels in serum and tissues due to ontogenic, hormonal and nutritional regulation, with GH being the most important postnatal stimulus for IGF-I production if energy supply is not reduced [26]. However, in human foetal serum, IGF-I levels are largely GH-independent and relatively low. During childhood, serum IGF-I levels rise gradually [26], whereas they rise steeply during puberty, peak clearly before the end of puberty and correlate with Tanner stages until peak height velocity is attained. Circulating IGF-I is partly either directly or indirectly related to the action of sex steroids [3, 26] (see also chapter 2.2.3). In adulthood, secretion of human GH progressively declines with age, accompanied by decreased levels of serum IGF-I [26].

The IGFBP family includes six distinct high-affinity binding proteins (IGFBP-1 to IGFBP-6). Under most conditions, the IGFBPs appear to inhibit IGF action. Additionally IGFBPs convey IGF independent functions including growth inhibition and direct induction of apoptosis [26]. In the following paragraphs, the roles of IGFBP-1 to IGFBP-3 will be described, since they are relevant for this thesis.

IGFBP-3 is the most abundant IGFBP in the blood, binding 70-90% of circulating IGF-I within a ternary complex comprising IGF-I, IGFBP-3 and an acid labile subunit [197, 205]. Throughout the day, IGFBP-3 levels are relatively stable and they do not respond acutely to

nutritional influences, however, chronic dietary restriction may decrease IGFBP-3 concentrations [206]. IGFBP-3 is well documented to inhibit cell growth and/or promote apoptosis, which is achieved through the attenuation of IGF-I/IGF-insulin receptor interaction. On the other hand, it has also been shown that IGFBP-3 stimulates cell growth and other cell functions independent of IGF-I [205]. It appears evident that IGFBP-3 limits the bioavailability of IGF-I [205]. **IGFBP-1** binds only a small fraction of circulating IGF-I. It fluctuates acutely in response to dietary and metabolic changes, increasing in the fasting state because of some inhibitory effects of insulin and stimulatory effects of cortisol and glucagon. IGFBP-1 decreases in the postprandial state with increased levels of insulin and glucose [206, 212]. High IGFBP-1 levels limit the acute IGF-I bioavailability and decrease the insulin-like activity of IGF-I on peripheral metabolism. By contrast, in certain situations IGFBP-1 can potentiate the effects of IGF-I on cellular responses because posttranslational modifications of the protein can diminish the affinity of IGFBP-1 for IGF-I, potentially releasing IGF-I in the circulation [212]. For instance, IGF-I was found to be involved in the process of dermal wound healing by stimulating reepithelialisation of the wounds and this action is potentiated by IGFBP-1 [213]. Furthermore, IGFBP-1 can induce IGF-I independent effects on cell signalling. Independent of IGF-I, IGFBP-1 seems to modulate insulin sensitivity by a putative mechanism involving stimulation of insulin signalling *via* the integrin–focal adhesion kinase–integrin-linked kinase–phosphatidylinositol-3-kinase–protein kinase B pathway and stimulation of glucose uptake in cells [212]. Similar to IGFBP-3, **IGFBP-2** levels are more stable than IGFBP-1 and do not respond to postprandial metabolic changes [206], but still seem to be dependent on nutrition [214]. Dietary restriction of protein was shown to increase IGFBP-2 levels in children and adults [215]. IGFBP-2 interaction with IGF-I possibly include the delivery of IGF-I to its target cells [212]. Independent of IGF-I, IGFBP-2 has been found to enhance cellular proliferation and decrease apoptosis also in the pathological state of cancer [216]. By both IGF-dependent and IGF-independent mechanisms, IGFBP-2 might potentially activates the focal adhesion kinase, which was shown to enhance skeletal insulin sensitivity in vitro [217] and might inhibit enzyme activity of a phosphatase and tensin homolog which has been shown to supress insulin signalling in adipocytes and skeletal muscle and glucose uptake [212]. Thereby IGFBP-2 may modulate insulin sensitivity in metabolically active tissues so that higher IGFBP-2 levels primarily reflect higher insulin sensitivity. In fact, IGFBP-2 concentrations mirror longer term insulin sensitivity [212].

Relevance of the GH-IGF axis

The GH-IGF axis plays a central role in cell growth, proliferation, differentiation and apoptosis [218], affecting nearly every organ system in the body. Furthermore, evidence of the relevance of the GH-IGF axis in the glucose metabolism is increasing. IGF-I has great structural homology to insulin and exerts similar metabolic actions such as glucose uptake in peripheral tissues [219]. In individuals with or without type 2 diabetes, administration of IGF-I has been shown to decrease glucose levels and improve insulin sensitivity [220-222].

Obesity

Besides other processes, IGF-I regulates adipose tissue growth and differentiation of pre-adipocytes into adipocytes [192] and elevated IGF-I levels may be related to later obesity, as described in chapter 2.2.1 (*Potential mechanisms relating glucose and insulin response to body composition and obesity*). Briefly, effects of IGF-I include the stimulation of cellular glucose uptake in pre-adipocytes and adipocytes as well as the possible increase of lipogenesis and inhibition of lipolysis in adipocytes [192-194]. Therefore, higher levels of IGF-I may contribute to body fat formation and thus in situations of abundant energy availability may contribute to the development of obesity. However, in humans, relations between IGF-I and obesity are contradictory. Studies have been conducted in different populations and age groups. Obese adults were found to have low or low-normal IGF-I levels, compared to normal weight adults, whereas normal to high IGF-I levels were reported for overweight children [214]. Among obese adolescent girls IGF-I decreased after weight loss [223]. On the other hand, higher animal protein intake or permanent essential amino acid supplementation improves lean body mass and basal muscle protein synthesis probably via an increased IGF-I protein expression [224]. IGF-I increases are also considered to stimulate muscle protein synthesis and glycogen storage [225].

Diabetes

Cross-sectional data suggest that insulin resistance is associated with changes in the GH-IGF axis. Among individuals with impaired glucose tolerance and type 2 diabetes elevated free IGF-I levels have been observed [226]. These individuals had also lower IGFBP-1 [227-229] and IGFBP-2 [226] levels compared to healthy controls. In contrast, higher IGFBP-3 levels were associated with higher fasting insulin levels [226, 230, 231]. Rajpathak et al speculated that low IGFBP-1, possibly low IGFBP-2, and high free IGF-I may represent compensatory mechanisms in response to increasing insulin resistance. High IGFBP-3 levels on the other hand may be a risk factor for insulin resistance and type 2 diabetes [232]. Cross-sectional

studies are however overall limited in their ability to assess causality of such relations. In fact, it is difficult to examine whether the diabetic state itself may be the cause of changes in the GH-IGF axis or whether it is the other way around, i.e. a result of impaired GH-IGF-action – a situation termed "reverse" causality [232].

Prospective cohort studies examining the relation between GH-IGF axis and type 2 diabetes are scarce. The only prospective cohort study among 615 healthy women and men aged 45-65 years investigated total IGF-I and IGFBP-1 levels [233]. Sandhu et al found an inverse association between IGF-I levels and the risk of developing impaired glucose tolerance or type 2 diabetes after an average of 4.5 years of follow-up. In particular, in adults with IGF-I levels above the median ($\geq$152μg/L) the risk of type 2 diabetes was only half the risk than that in participants with IGF-I levels below the median. In addition, an inverse association between total IGF-I levels at baseline and the 2-hour post-load glucose concentration measured at the end of follow-up was observed, but only among participants with low IGFBP-1 concentrations ($\leq$25μg/mL) [233].

It has been proposed, that in a state of insulin resistance, circulating IGF-I helps maintaining euglycaemia by influencing peripheral glucose uptake by IGF-I receptors. If insulin resistance becomes worse, an increased expression of insulin/IGF-I hybrid receptors helps to increase the glucose uptake in muscle as well as adipose tissue [232]. Furthermore, insulin like effects of IGF-I together with the increase in insulin/IGF-I hybrid receptors result in an uptake of free fatty acids, reducing the negative impact of free fatty acids on insulin sensitivity and their lipotoxic effect on β-cells [13, 234]. With the worsening of insulin resistance insulin levels increase, resulting in lower serum IGFBP-1 levels, up-regulation of hepatic IGF-I production, and higher levels of free IGF-I levels. Additional possible mechanisms might work through the influence of the GH-IGF axis to preserve β-cell mass and function as well as the anti-inflammatory effect of IGF-I, reducing inflammatory cytokine levels and thereby affecting insulin resistance and the progression to type 2 diabetes [232]. Higher IGF-I may therefore reduce the risk of type 2 diabetes.

Cardiovascular disease

Moreover, higher IGF-I levels have been prospectively related to lower risks of heart failure, ischaemic heart disease and stroke [235-238]. In the cardiovascular system, IGF-I is postulated to protect against endothelial dysfunction, atherosclerotic plaque development, and ischemic myocardial damage. Some of these effects are related to nitric oxide production, induced by IGF-I. The release of nitric oxide has multiple metabolic and vascular-protective

effects such as vasodilation, antiplatelet actions or endothelial cell migration, proliferation, and survival [239].

Cancer

Evidence from mouse models, animal and human cancer cell lines showed that GH, IGF-I, and insulin can stimulate and contribute to cancer progression [240]. An inappropriate expression of the GH-IGF axis appears to contribute to the growth, maintenance and progression of the most common cancers, including breast, lung and colon cancers [241]. Biological literature has stressed mitogenic and anti-apoptotic effect of IGF-I hence promoting tumorigenesis [242]. Because IGFBP-3 binds IGF-I it regulates the bioavailability of IGF-I and may thus contribute to a reduced cancer risk. Independent of IGF-I, IGFBP-3 has also antiproliferative and pro-apoptotic effects which may explain inverse associations between IGFBP-3 concentrations and cancer risk [205, 242].

Prospective studies were conducted to assess the relation between the GH-IGF axis and cancer incidence to provide direct evidence of cancer risks in healthy individuals. Meta-analyses of prospective data [243-247], including pooled analyses of individuals patient's data [244, 247], suggest that raised IGF-I levels are associated with a slightly increased risk of some cancers: In fact, high IGF-I levels have been related to an increased risk of prostate [245, 247], breast [244], and colorectal cancer [245, 246]. No relations were found between IGF-I and lung cancers [243, 245]. Moreover, meta-analyses examined the relation between IGFBP-3 and cancer risks: Increased IGFPB-3 levels have been found to be associated with higher risks of prostate cancer [247] and breast cancer in postmenopausal, but not premenopausal women [244]. While associations between IGF-I and risk of prostate cancer [247] or breast cancer [244] were unaffected by adjustment for IGFBP-3 levels, the associations between IGFBP-3 and cancer risk were eliminated in these studies by adjustment for IGF-I, hence, questioning a direct association between IGFBP-3 and cancer. In line with a probable anticancer potential, increased IGFBP-3 seemed to be related to a reduced risk of lung cancer [243], this association could nevertheless be confounded by smoking status because current smoking had been associated with significant reductions in mean IGFBP-3 levels [248]. In addition, recent evidence from a case-control study showed that IGFBP-3 were lower in women with breast cancer [249]. Thus, increased IGFBP-3 concentrations may not be as detrimental as it has been suggested.

2.2.3 Concept of critical periods

The concept of critical periods for obesity development has been proposed by Dietz [1, 250], defining them as "developmental stage in which physiologic alterations increase the risk of later obesity" [250]. The four critical periods include the prenatal period, the early postnatal period, the period of adiposity rebound, and puberty [1, 2]. There exists the possibility that nutritional alterations during critical periods of development lead to adverse health conditions, such as adult obesity [1]. Early life, adiposity rebound, and puberty are relevant phases for this thesis and will be described subsequently.

Early life

Research on the relevance of early life factors for later disease risk, a concept often referred to as programming, has mainly focussed on rapid infancy weight gain and breastfeeding [251, 252]. Breastfeeding is now considered to result in a small, but protective effect against childhood obesity [253, 254], which could be due to the lower protein content in human milk compared to formula [255]. According to the "early protein hypothesis", high protein intake with infant formula feeding, in excess of metabolic requirements, might induce increased circulating concentrations of insulin-releasing amino acids, which in turn might stimulate the secretion of IGF-I, thereby inducing an increased weight gain during the first 2 years of life as well as increased adipogenic activity [255]. In this regard, protein intake has also received attention under the consideration that higher protein intake provided by formulas may stimulate insulin release and the GH-IGF axis [256], leading to increased weight gain as has been shown recently [257]. In addition, studies which examined the importance of protein intake on the GH-IGF axis indicated inverse associations between milk intakes in early life and IGF-I levels in adulthood (see also chapter 2.3.3) [31, 32]. This inverse relation reflects an early programming of the GH-IGF axis in response to higher protein intakes in early life. To date, no data is available on the relevance of animal protein, including meat and dairy protein, as well as plant protein intakes in early life and body composition or the GH-IGF axis. In this context, the relevance of insulin demand in early life would also be of interest as increased insulin levels in early life may play a role in the development of obesity [255]. However, there are no published GI or FII values available for human breast milk or formulas thus far and only four published GI values for baby foods [52]. Therefore, analyses included in this thesis could only consider dietary protein intake when examining relations of dietary factors during early life with body composition and the GH-IGF axis.

Adiposity rebound

Another potentially critical period is the time around the adiposity rebound. After birth, changes in BMI occur and they can be displayed in characteristic growth curves: After a steep increase in the first months of life, growths peaks around 12 months of age, followed by a subsequent decrease, reaching the lowest point before the second and final increase in growth to reach adult levels. The adiposity rebound corresponds to the turning point before the second rise in the BMI curve [258]; in Germany, the mean age at adiposity rebound is around 4.5 years for girls and 5 years for boys [92] (**Figure 2**).
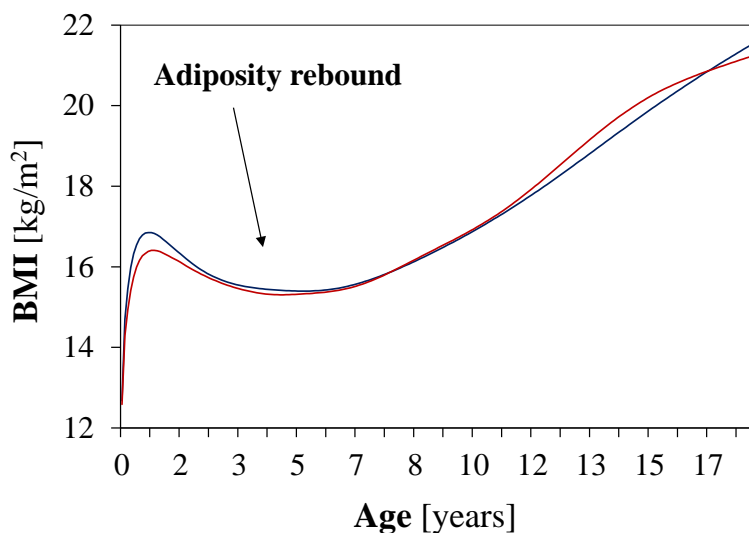
Figure 2 Adiposity rebound in German girls (red) and boys (blue) with a BMI corresponding to the 50th BMI percentile [92]

Rolland-Cachera et al first suggested that an early adiposity rebound might represent a risk factor for obesity later in life [259]. Since then, several analyses have confirmed an inverse association between the age at adiposity rebound and BMI later in childhood as well as adulthood [260-262]. Later on, it was discovered that differences in BMI during adiposity rebound were caused specifically by alterations in body fat and not by alterations in lean mass or height. Children undergoing early adiposity rebound gained fat at a faster rate than children who rebounded at a later age [263] which eventually justified using the term "adiposity rebound" [264]. In addition, an early adiposity rebound was found to be a risk factor for type 2 diabetes [265, 266]. Studies have also demonstrated that a higher BMI at adiposity rebound seems to increase subsequent obesity risk too [262, 267]. Consequently, debate continued whether the age or the BMI at adiposity rebound predicts fatness later in life. It has been hypothesised that an early adiposity rebound represents a growth pattern different from the one associated with a high BMI at adiposity rebound, but both result in a higher risk of

obesity later in life. Children with an early adiposity rebound have normal or even low BMI levels before the adiposity rebound and show higher body fatness after the adiposity rebound, whereas children with a high BMI at adiposity rebound probably had a high BMI at all ages before the adiposity rebound [258]. Results from the DONALD study displayed an association between a higher habitual protein intake between the age of 12 and 24 months and higher BMI SDS values at adiposity rebound among girls only, but no consistent relation between habitual protein intake in early childhood and timing of adiposity rebound was found [268]. Furthermore, dietary habits such as high intakes of vegetable and animal protein during the years before puberty may influence pubertal timing [269], supporting a specific relevance of dietary intake during the potentially critical period of adiposity rebound.

Puberty

Puberty represents the last period discussed to be potentially critical for later disease risk, including the risk of adverse changes in body composition, obesity [1] and type 2 diabetes [270]. Overall, this period of growth and maturation is marked with behavioural changes in diet, physical activity, sedentary behaviour and psychological health [271]. Puberty is also characterized by changes in levels of IGF-I, growth hormones and sex steroids [4] as well as a physiological insulin resistance. In fact, IGF-I levels rise steeply during puberty and peak before the end of puberty, while the development of the insulin sensitivity follows the reverse course (**Figure 3**, see also chapter 2.2.2) [3, 272]. The fall in insulin sensitivity during puberty is not related to body fat content, but due to increases in growth hormone, which is also known to increase rates of lipolysis in the liver and elevate circulation of free fatty acids [3, 271]. Lower insulin sensitivity indicates a greater insulin resistance: Compared to males, females have greater insulin resistance during puberty [3]. Furthermore, the physiological insulin resistance is characterised by higher levels of both fasting glucose and insulin, while the acute insulin responses are disproportionally low. This suggests either a conservation in β-cell function or an inadequate β-cell response because β-cells do not fully adapt to the decrease in insulin sensitivity [272]. However, higher postprandial glycaemic and insulinaemic excursions result in greater demands on β-cell function which may lead to exhaustion of β-cells. In this context, specifically the relevance of dietary insulin demand, but also protein intake, is of interest in this critical period with regard to development of body composition and type 2 diabetes [273].
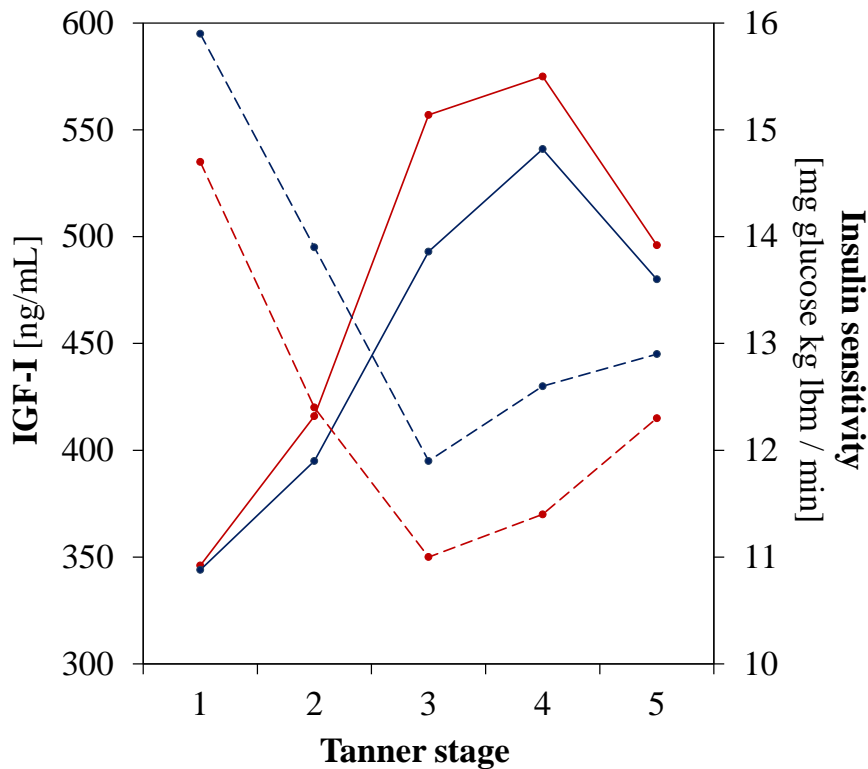
Figure 3 Changes in IGF-I levels (solid line) and insulin sensitivity (dashed line) from Tanner stages 1 to 5 in females (red) and males (blue). A lower insulin sensitivity value indicates greater insulin resistance. lbm, lean body mass (after Moran et al [3])

2.3 Evidence linking dietary insulin demand and protein intake to body composition, obesity and GH-IGF axis

2.3.1 Glycaemic index, glycaemic load, body composition and weight loss

There exists controversy concerning the role of dietary GI and/or GL for the development of body composition and obesity; potential mechanisms have been described earlier (see chapter 2.2.1). Prospective cohort studies suggest a detrimental role of high dietary GI [162, 274-276] and GL [276] for body composition among women, while only one study reported an association between a higher dietary GI and higher waist circumference among men [276]. By contrast, a cross-over intervention study did not observe an effect of dietary GI on changes in body weight, waist circumference or %BF among overweight and obese women [277]. Regarding weight loss, reviews concluded that among overweight or obese adults, low-GI/GL diets are as effective as other dietary alternatives [21]. However, little is known about the role of dietary GI or GL in the development of obesity among children and adolescents as well as about an optimal dietary approach for weight loss in overweight or obese children and

adolescents. Among children and adolescents, four observational studies, six intervention studies including two retrospective analyses of two interventions and four clinical trials were identified which examined the relation between dietary GI and/or GL (in childhood and adolescence), body composition (BMI, fat mass, %BF, waist and hip circumference, waist to hip ratio) and weight loss (**Table 2** and **Table 3**).

Observational studies

Four prospective studies among healthy children and adolescents in Germany [278, 279] and Australia [280] and overweight Latino adolescents in the US [281] were identified, which examined dietary GI and or GL and body composition (**Table 2**).

Among 856 healthy school children aged 12 years at baseline, Gopinath et al did not find a prospective relation between GI or GL at baseline and change in BMI, %BF or waist circumference at 5 year follow-up. However, among girls (n=421), an increase of 1 SD GL at baseline was significantly associated with a concurrent increase in BMI and waist circumference at follow-up [280]. In two prospective analyses of the DONALD study, no association between changes in GI or GL and simultaneous development of BMI and %BF were found for either childhood (2-7 years; n=380) [278] or adolescence (8.7-12.7 years in girls; n=116 and 10.3-14.3 years in boys; n=99) [279]. However, overweight adolescents with a higher dietary GI at baseline tended to have higher %BF and BMI SDS at baseline, while no association was observed for normal weight adolescents [279]. Among 85 overweight Latino adolescents aged 14 years at baseline, changes in GI or GL during a 2-year follow-up were not associated with changes in BMI, total body fat or with changes in the visceral or subcutaneous adipose tissue, respectively [281].

Prospective studies do not draw a clear picture for healthy as well as overweight children and adolescents and they do not support a strong relation between dietary GI and/or GL in the development of body composition.

Table 2 Observational studies in children and adolescents examining the relation between dietary GI and GL and adiposity measures[1]

Author	Design and population	Exposure and Outcome	Results
Gopinath et al 2013 [280] Australia	• Population-based survey (Sydney Childhood Eye Study); started between 2004 and 2005; prospective and concurrent analyses • n=856 (435 males) with complete 5 year follow-up data • 12 years of age at baseline	**Exposure:** Dietary GI and GL at baseline **Mean (SD) dietary GI** at baseline: girls: 54 (3), boys: 54 (3) **Mean (SD) dietary GL** (g/d) at baseline: girls: 138 (SD: 53), boys: 145 (54) **Assessment method:** 120 item self-administered FFQ **Outcome:** BMI, %BF (BIA), and waist circumference (measured at baseline and 5 years) **Covariates:** Age, sex, ethnicity, parental education, exposure to passive smoking, change in height, screen viewing time, time spent in physical activity, and energy intake	• **Among girls**, **no prospective relation** between dietary **GI** and **BMI, %BF or waist circumference** (all p for trend >0.1) and a **prospective trend** between dietary **GL** at baseline and **change in BMI, %BF, but not waist circumference** (p for trend were 0.08, 0.07 and >0.9, respectively) • Each 1 SD increase in dietary **GL** was **associated with a concurrent increase in BMI and waist circumference** (0.77 kg/m^2 and 1.45 cm, respectively; both p for trend=0.01), **but not %BF** (1.1%; p for trend=0.18) in girls • Each 1 SD increase in dietary **GI tended** to be associated with a **concurrent increase in BMI and waist circumference** (0.28 kg/m^2; p for trend=0.09 and 0.40 cm; p for trend=0.08), but not %BF (0.61%; p for trend=0.15) in girls • **Among boys**, **no relation** between dietary GI or GL and change in anthropometric measures were found (analyses of prospective and concurrent associations)
Buyken et al 2008 [278] Germany	• Ongoing open cohort study (DONALD study); started in 1985; cross-sectional, prospective, and concurrent analyses • n=380 (203 males) healthy term singletons with complete dietary and anthropometric data at least at ages 2 and 7 years	**Exposure:** Dietary GI and GL between 2 and 7 years of age: **Mean (SD) dietary GI** was 52 (4) at age 2 years and 56 (3) at age 7 years **Mean (SD) dietary GL** (g/d) was 63 (15) at age 2 years and 113 (24) at age 7 years **Assessment method:** 3-day weighed dietary records **Outcomes:** %BF (estimated using skinfold measurements) and BMI SDS (measured at 2 and 7 years of age) **Covariates:** Age, age^2, age^3, sex, maternal	• Overall, dietary **GI or GL** were **not related** to %BF or BMI SDS (analyses of cross-sectional and concurrent associations) • **Trend** for prospective associations between **GI**, but not GL, and **%BF** (p for trend were 0.07 and 0.4, respectively) • **Trend** for prospective associations between **GL**, but not GI, and **BMI SDS** (p for trend were 0.07 and 0.2, respectively)

Table 2 *continued.*

Author	Design and population	Exposure and Outcome	Results
Buyken et al 2008 *continued*		overweight, year of birth, birth weight, rapid weight gain between birth and age 2, intakes of energy, protein, fibre, and added sugar	

Cheng et al 2009 [279] Germany	• Ongoing open cohort study (DONALD study); started in 1985; cross-sectional and concurrent analyses
	• n=215 (99 males) healthy term singletons with complete dietary and anthropometric data around the time of puberty onset (defined by age at take-off)
	• Overall mean age (SD) at baseline and endpoint: 9.4 (1.2) and 13.4 (1.2) years, respectively
		Exposure: Dietary GI and GL at baseline and endpoint
		  Mean (SD) dietary GI was 56 (3) at baseline and 57 (4) at endpoint
		  Mean (SD) dietary GL (g/d) was 120 (30) at baseline and 152 (43) at endpoint
		Assessment method: 3-day weighed dietary records
		Outcomes: %BF (estimated using skinfold measurements) and BMI SDS (measured at baseline and endpoint)
		Covariates: Age, age^2, age^3, sex, maternal overweight, breastfeeding, energy, and fibre intake
			• Dietary **GI or GL** were **not related to %BF or BMI SDS** throughout puberty (analyses of cross-sectional and concurrent associations)
			• **Overweight adolescents** with a **higher dietary GI at baseline tended to have higher %BF** (p for trend=0.05) **and BMI SDS** (p for trend=0.01) at baseline, while no association was observed for normal weight adolescents (p for interaction at baseline: 0.04 for %BF and 0.07 for BMI SDS)
			• Estimates of the **concurrent association among overweight adolescents** analysis **did not reveal any associations** (p for interaction for concurrent association: 0.03 for %BF and 0.08 for BMI SDS)

Davis et al 2009 [281] US	• Prospective cohort study (Study of Latino Adolescents at Risk for Diabetes cohort); started in 2000 • n=85 (48 males) overweight Latino adolescents with 2 complete annual visits • Mean age (SD) at baseline: 14.2 (1.6) years • Mean follow-up (SD): 1.5 (0.5) years	**Exposure:** Dietary GI and GL at baseline At baseline, **mean dietary GI** (SD) was 59 (6) and **mean dietary GL** (g/d) was 133 (51) **Assessment method:** Multiple pass 24h dietary recalls **Outcomes:** Whole body fat, soft lean mass tissue (DEXA), subcutaneous abdominal adipose tissue and visceral adipose tissue (MRI), glucose, and insulin indexes (OGTT) (measured at baseline and follow-up) **Covariates:** Sex, Tanner stage, time between visits, baseline visceral adipose tissue, energy and fibre intake, and baseline subcutaneous abdominal adipose tissue	• Dietary **GI or GL** were **not related** to changes in adiposity variables or changes in glucose and insulin indexes

[1] Only relevant exposures and outcomes are presented; Adjusted p-values are presented, if not indicated otherwise

Clinical trials

Two retrospective studies of a 3 [282] and 4 months [283] intervention among obese children in the US were identified. Furthermore, four randomised controlled diets were found of which three were conducted in the US and examined the effect of low-GI and/or GL diets compared to low fat [284, 285] or portion controlled and low carbohydrate diets [286] among overweight and/or obese children and adolescents. Intervention periods were 3 months [286], 6 months [284], and 2 years [285], respectively. The forth study is the DIOGenes study, an European multicentre, randomized, controlled 6 month intervention study investigating the effects of dietary protein and glycaemic index on weight (re)gain in obese and overweight families. Families with parents who lost >8% of their body weight during an 8 week run-in low-calorie diet period were randomly assigned to 1 of 5 *ad libitum* diets (i.e. a low protein (LP)/low glycaemic index (LGI), LP/high-GI (HGI), high protein (HP)/LGI, HP/HGI or a control diet). It should be noted that this study primarily focussed on the obese/overweight parents and hence children included in the study represent a group at risk of overweight with a baseline prevalence of obesity/overweight of 47.5% (**Table 3**).

The first retrospective study found that among 11 year old participants, those on a low-GI Healthy Eating Plan (HEP), additionally supported by a dietician, decreased their BMI (n=21) compared to participants on a HEP without support by a dietician (n=15) or on a portion controlled diet (n=28) [282]. Similarly, among 10 year old participants those in the low-GI group (n=64) had greater decreases in their BMI and weight compared to participants in the low fat group (n=43). However, the macronutrient content of the diets was not matched and results cannot be attributed to GI only [283].

The clinical trial of Ebbeling et al found that after a 6 month intervention and 6 months follow-up, BMI and fat mass had decreased more in the low-GL diet group compared to the low fat diet group among 16 obese adolescents aged 13-21 years [284]. Among 113 obese Hispanic children aged 7-15 years, a 2 year intervention showed that a low-GL and a low fat diet were equally effective for reduction of BMI [285]. Likewise, a low-GL diet was found to be as efficient as a standard portion-controlled or low carbohydrate diet for weight management in a 3 month intervention among 102 obese children aged 7-12 years [286]. The analysis within the DiOGenes study included 465 children aged 12 years at baseline. The dietary GI did not have an isolated effect on body composition, but that the LP/HGI diet was related to an increase in %BF and that the HP/LGI was related to a reduction in the percentage of overweight and obese children after 6 months of intervention [25].

Retrospective studies suggest an association between GI and adiposity measures among obese children. Among the randomised controlled trials, only Ebbeling et al showed differences in BMI and body fat between the reduced GL and control diet [284], but with 16 participants and only 14 completing the study this result is rather weak. Overall, data available to date does not support a strong role of dietary GI or GL for the development of body composition during childhood or adolescence. However, the combination of a lower GI diet with higher protein content may offer some benefits for children or adolescents at risk of overweight.

Table 3 Clinical trials in children and adolescents examining the relation between dietary GI and GL, weight loss and adiposity measures[1]

Author	Design and population	Exposure and Outcome	
Siegel et al 2011 [282] US	• Retrospective study of a 3 months intervention • Obese children assigned to a Healthy Eating Plan (HEP; assignment started in 10/2009), including a visit with a dietician; parents decide whether child stayed in HEP or switched to portion controlled diet (PC) • Complete data was available for n=64 participants (36% males) who were following a portion controlled diet (PC; n=28), a HEP (n=21), and a HEP, but not seeing a dietician (n=15) • Mean age (SD): 11.3 (3.3) years	**PC** and dietician **HEP** and dietician **HEP** no dietician HEP: <10% of food items calories are saturated fat and low-GI ($\leq$ 50) **Mean GI or GL of the diets were not reported** **Outcome:** BMI (measured at baseline and 3 months) **Covariates:** n/a	• Participants within **HEP supported by a dietician** had a significantly **larger decrease in BMI** from baseline to 4 months follow-up (-1.25 kg/m^2) compared to the other groups (PC: -0.2 kg/m^2 and HEP no dietician: +0.4 kg/m^2; unadjusted p for difference<0.001)
Spieth et al 2000 [283] US	• Retrospective study of a 4 months intervention • Obese children assigned to a weight loss program (09/1997 to 12/1998), based on schedule availability, i.e. a low-GI n=64 (30 males) or reduced fat diet n=43 (19 males) with complete data • Mean age (SD) of children consuming a low-GI and reduced fat diet was 10.6 (4.0) and 10.2 (3.1) years, respectively	**Low-GI/GL diet**, *ad libitum* (45-50%En carbohydrate, 20-25%En protein, 30-35%En fat) **Reduced fat diet** (55-60%En carbohydrate, 15-20%En protein, 25-30%En fat) **Mean dietary GI or GL of the diets were not reported** **Outcome:** Weight and BMI (measured at baseline and last visit) **Covariates:** Age, sex, ethnicity, duration of follow-up, behavioural therapy referral, and baseline BMI	• Participants on the **low-GI diet** had a **larger decrease in BMI** (-1.15 kg/m^2 (95% CI -1.69, -0.60) vs. -0.03 kg/m^2 (-0.51, 0.57); p for difference=0.001) **and body weight** (-1.16 kg (-2.64, 0.33) vs. 1.44 kg (-0.03, 2.91); p for difference=0.007) than those on the reduced fat diet • A larger percentage of participants in the low-GI diet had a decrease in BMI of at least 3 kg/m^2 compared to participants in the reduced fat diet (11 participants (17.2%) vs. 1 participant (2.3%); p for difference=0.03) • Macronutrient content of the diets was not matched and results cannot be attributed to GI only

Table 3 *continued.*

Author	Design and population	Exposure and Outcome	Results

Ebbeling et al 2003 [284] US	• Randomised controlled trial; started between 12/2000 and 09/2001
	• 6 months intervention, 6 months follow-up
	• Obese adolescents (n=16 (5 males)) randomised to either an *ad libitum* reduced GL diet (RGL) or energy restricted, reduced fat diet (RFD)
	• 13-21 years of age
	• N=14 completed the study, 7 participants in each diet group
		Randomisation groups:
		RGL (45-50%En carbohydrate, 30-35%En fat)
		    Mean (SEM) dietary GI and GL (g/1000kcal) were 53 (3) and 69 (6), respectively
		RFD (55-60%En carbohydrate, 25-30%En fat; negative energy balance of 250 to 500 kcal/day)
		    Mean (SEM) dietary GI and GL (g/1000kcal) were 56 (2) and 79 (7), respectively
		Outcome: BMI, %BF, and fat mass (DEXA) (measured at 0, 6, and 12 months)
		Covariates: Energy intake, change in BMI
			• At 12 months, **BMI and fat mass had decreased significantly more in the RGL** compared with RFD (mean (SEM): −1.3 (0.7) vs. 0.7 (0.5) kg/m^2; p=0.02 and −3.0 (1.6) vs. 1.8 (1.0) kg; p for difference=0.01)
			• Decrease in BMI (p for difference=0.03) and fat mass (p for difference=0.02) from 0 to 12 months in RGL group
			• No changes in the RFD group

	• Pooled analysis using data from both diet groups	**Exposure:** Change in GL and fat intake (baseline to 6 months) **Assessment method:** 7-day food diaries	• **Change in GL** (g/1000kcal) was a **strong predictor of change in %BF** (0-6 months), explaining about half of the variance in both groups combined (R^2=0.51; p for trend=0.006)

Mirza et al 2013 [285] US	• Randomised controlled trial; started between 11/2003 and 05/2008
	• 2-year intervention (from baseline on, 12 weeks of nutrition education and dietary counselling sessions)
	• Hispanic, obese, otherwise healthy children randomised to either a low-GL diet (LGD; n=57 (25 males)) or low fat diet (LFD; n=56 (33 males))
	• n=33 (18 males) and n=31 (18 males) completed the 2 year follow-up
	• 7-15 years of age
		Randomisation groups:
		LGD, *ad libitum* (45-50%En LGI carbohydrate, 20-25%En protein, and 30-35%En fat)
		    Mean (SE) dietary GI was 51 (1) after 3 months and 56 (1) after 2 years
		    Mean (SE) dietary GL (g/1000kcal) was 64 (3) after 3 months and 77 (4) after 2 years
		LFD (55-60%En carbohydrate (with no discrimination by GI), 15-20%En protein, and 25-30%En fat; moderate decrease in caloric intake (500-1,000 kcal/day) [287])
		    Mean (SE) dietary GI was 55 (1) after 3 months and 54 (2) after 2 years
		    Mean (SE) dietary GL (g/1000kcal) was
			• **No differences** in averaged mean **BMI z-score** at any of the measured time points (all p for difference>0.1) or the decrease in overall BMI z-score **between LGD and LFD** (controlled for baseline BMI z-score; p for difference=0.8)
			• Both dietary groups decreased their BMI z-scores at 3, 12 months, and 2 years post-intervention (p for difference were <0.0001, 0.003, and 0.002, respectively; using multiple imputation as well as completers-only analyses)

Table 3 *continued.*

Author	Design and population	Exposure and Outcome	Results
Mirza et al 2013 *continued*		73.8 (2.5) after 3 months and 73.6 (3.4) after 2 years **Assessment method:** 24h dietary recall and 2-week dietary recall **Outcome:** BMI z-score (measured at baseline, 3, 12 months, and 2 years) **Covariates:** Baseline BMI z-score	

Kirk et al 2012 [286]
 	
US
	• Randomised clinical trial; started in 03/2005
	• 3 months intervention
	• Obese children randomised to one of the following diet groups: Low carbohydrate diet (LC; n=35 (16 males)), reduced GL diet (RGL; n=36 (10 males)) or portion controlled, energy restricted diet (PC; n=31 (8 males))
	• Dietary counselling for the first 12 weeks
	• 7-12 years of age
	• N=85 completed the 12 months assessment
		Randomisation groups:
		LC (2-week induction phase with ≤20 g carbohydrate/day, increased by 5-10 g/week up to a maximum of 60 g/day, no limit on intake of high protein foods and fats)
		    At baseline, **mean (SD) dietary GL** (g/1000kcal) was 73 (11)
		RGL (limited intake of high-GI foods, no restrictions on protein or fat intake)
		    At baseline, **mean (SD) dietary GL** (g/1000kcal) was 77 (11)
		PC (55%-60%En carbohydrate, 10%-15%En protein, and 30%En fat; daily 500kcal deficit relative to each subject's expected energy requirement)
		    At baseline, **mean (SD) dietary GL** (g/1000kcal) was 74 (13)
		Outcome: BMI z-scores, %BF (DEXA), waist circumference, insulin, and glucose (measured at baseline and 3, 6, and 12 months)
		Covariates: Energy intake, change in BMI z-scores
			• **No differences** in BMI z-scores, %BF or waist circumference **between the diet groups**
			• In all diet groups, BMI z-scores and %BF decreased after 3 months of intervention (all p for difference were ≤0.0001 and ≤0.0002, respectively), remaining reduced through to 12 months (all comparisons with baseline p for difference were ≤0.0001 and ≤0.0002, respectively)
			• Waist circumference was lower than baseline at 3 and 6 months (both p for difference≤0.0001), but not 12 months (p for difference≥0.08)

Table 3 *continued.*

Author	Design and population	Exposure and Outcome	Results
Papadaki et al 2010 [25] Europe: Netherlands, Denmark, UK, Greece, Germany, Spain, Bulgaria, and Czech Republic	• Analysis within an randomised 6 months intervention trial, a family based study (DiOGenes; started between 11/2005 and 04/2007) • Families with parents who lost >8% of their body weight during an 8 week run-in low-calorie diet period were randomly assigned to 1 of 5 ad libitum diets • n=465 (201 males) children with complete data were allocated to a low protein (LP)/low glycaemic index (LGI; n=102), LP/high-GI (HGI; n=87), high protein (HP)/LGI (n=92), HP/HGI (n=96) or a control diet (n=88) • Mean age (SD) at baseline: 12.4 (3.5) years in girls and 11.9 (3.4) years in boys	Randomisation groups: **LP/LGI, LP/HGI, HP/LGI, HP/HGI, Control diet** **All diets** were *ad libitum* and low in fat (25%-30%En); target protein intake was 10%-15%En for LP and 23%-28%En for HP groups (accepted range 10%-30%En protein) **Aim** to achieve a **15 GI point difference** and **13 protein percentage points** between LGI and HGI groups, respectively **Assessment method:** 3-day weighed dietary records (at baseline, 4 and 26 weeks) **Outcomes:** Differences in GI and protein intake between GI and protein groups BMI, %BF (DEXA), waist and hip circumference, and waist to hip ratio (measured at baseline, 4 and 26 weeks) **Covariates:** Age, gender, family structure, and country	• **No differences** in changes **in outcome measures** among the dietary groups during the intervention • **Increase in %BF in the LP/HGI group** over 26 weeks (1.53%; p for difference=0.04) compared to the other groups • **Reduction in the percentage of overweight/obese children in the HP/LGI group** over 26 weeks (-6.6%; p for difference=0.03) compared to the other groups • The **achieved differences between the GI and protein groups** were 2.3 GI points (p for difference=0.01) and 4.9 protein percentage points (p for difference<0.001), respectively. Hence the intervention was not successful in achieving the recommended 15 GI point difference between LGI and HGI groups

[1] Only relevant exposures and outcomes are presented; Adjusted p-values are presented, if not indicated otherwise

2.3.2 Protein intake, body composition and obesity

Besides the dietary GI and GL, dietary protein intake might be related to body composition (see chapter 2.2.1). Prospective studies in adults propose an unfavourable effect of dietary (animal) protein intake on body weight and BMI (but not specifically FMI or %BF) [288-291], whereas intervention studies in adults suggest that higher dietary protein intakes might have beneficial effects on weight loss, weight control and maintenance of fat-free mass, at least in the short-term [7, 19, 24, 292-295]. These results are controversial; however, it needs to be kept in mind that body weight and BMI are not the best proxy measures for body fat (see chapter 2.2.1). In fact, a recent randomized controlled trial examined that weight regain due to overeating protein resulted from gain in fat-free mass only [296]. Furthermore, protein intake is important during growth. As already mentioned, high protein intake with infant formula feeding, in excess of metabolic requirements, might increase weight gain during the first 2 years of life and increased adipogenic activity [255]. Among children and adolescents, four observational studies and six clinical trials were identified which examined the relation between dietary protein intake (in childhood and adolescence) – in specific dairy and meat products – and body composition (BMI, FMI and fat mass, FFMI and lean body mass, and %BF) and weight loss (**Table 4** and **Table 5**).

Observational studies

Four observational studies with a prospective design were identified, which investigated the association between dietary protein intake (%En) and body composition. Two studies were conducted in Denmark, including primary school-aged, healthy children who were followed-up until adolescence [297, 298]. The other two studies included healthy children and/or adolescents who were followed-up for 3 years in the US [299] and until young adulthood in the Netherlands [300] (see **Table 4**).

Two analyses of the Danish cohorts stratified their results according to participants BMI, i.e. quartiles of their BMI. The first study included 328 healthy children aged 6-7 years and found that only among lean girls (1-4th quantile of BMI), protein intake was inversely related to changes in FMI after 6 years of follow-up [298]. The second Danish study including 203 children aged 6-7 years observed that protein intake was inversely associated with changes in FMI after 3 years follow-up among heavier girls only (5th quantile of BMI) [297]. In addition, the first study showed a direct relation between protein intake and changes in FFMI in heavier girls (5th quantile of BMI) [298]; the second study could not confirm this [297]. While the relation between higher dietary protein intake and lower FMI was found once among lean and

once among heavier girls, the relation between higher protein intake and higher FFMI was only found among heavier girls. This may suggest that protein intake may have a beneficial effect on body composition specifically among heavier girls. Overall, these results are largely confined to females as no relation between dietary protein intake and FMI or FFMI was found in boys [297, 298]. This could result from differences in body composition development during puberty [298].

Berkey et al examined the relation between milk consumption and concurrent changes in BMI among 12,829 children and adolescents aged 9-14 years. Milk intakes were related to small, concurrent increases in BMI per year, which were specifically seen for milks with a lower fat content (skim milk among females and 1% milk among males). However, adjustment for energy intake attenuated the relation between intakes of skim milk and concurrent BMI change per year in females towards non-significance. Multivariate analyses suggested that total energy intake was the most important predictor for weight gain [299]. A Dutch cohort including 350 adolescents showed that dietary protein intake at the age of 13 years was not prospectively related to the sum of four skinfolds at the age of 36 years. Nevertheless, women with a >35%BF had about 1% lower protein intakes at the age of 13 years compared to women with lower %BF. These relations were not observed in men [300].

Taken together the results from observational studies there may not exist a direct relation between protein intake and an unfavourable body composition among children and adolescents. However, there is evidence available that protein intake may have a specific relevance among girls. Among heavier girls, protein intake may be related to a higher lean and lower fat mass.

Table 4 Observational studies in children and adolescents assessing the relation between dietary protein intake and adiposity measures[1]

Author	Design and population	Exposure and Outcome	Results
van Vught et al 2009 [298] Denmark	• Data from a prospective cohort study (The European Youth Heart Study); started in 1997 • n=328 (148 males) with complete baseline (8-10 years of age) and follow-up (14-16 years of age) measurements • 6 year follow-up	**Exposure:** Dietary protein (%En) at baseline Among **lean boys and girls** (1-4th BMI quintiles), **mean (SD) protein intakes** were 72.0g (21.3) and 69.2g (18.8), respectively (accounting for 12.9%En and 13.1%En, respectively) Among boys and girls in the **5th BMI quintile, mean (SD) protein intakes** were 70.6g (21.8) and 62.3g (20.0), respectively (accounting for 12.8%En and 13.1%En, respectively) (%En was estimated from table 1) **Assessment method:** 24h dietary recall **Outcome:** 6 year change in body composition: Fat-free mass index (FFMI) and fat mass index (FMI) (estimated using skinfold measurements; measured at baseline and follow up) **Covariates:** Baseline FMI or FFMI, age, energy intake, physical activity, SES and puberty stage	• Results were stratified according to BMI quintiles • Only among girls with a BMI in lower quintiles (1-4th) (i.e. **lean girls**), **protein** intake was **inversely associated** with **changes in FMI** (-1.22 ± 0.56; p for trend=0.03) • Only among **girls** with a **BMI in the 5th quintile**, protein intake was **directly associated** with **changes in FFMI** (3.99 ± 1.87; p for trend=0.04) • **No associations** were found **in boys**
van Vught et al 2010 [297] Denmark	• Data from a prospective cohort study (Copenhagen School Child Intervention Study); started in 2000 • n=203 (94 males) with complete baseline (6-7 years of age) and follow-up (9-10 years of age) measurements • 3 year follow-up	**Exposure:** Dietary protein (%En) at baseline Among **lean boys and girls** (1-4th BMI quintiles), **mean (SD) protein intakes** were 69.5g (17.8) and 63.4g (15.2), respectively (accounting for 14.2%En and 14.0%En, respectively) Among boys and girls in the **5th BMI quintile, mean (SD) protein intakes** were 71.3g (15.7) and 59.7g (13.7), respectively (accounting for 14.8%En and 13.5%En, respectively) (%En was estimated from table 1) **Assessment method:** Booklet for dietary record	• Results were stratified according to BMI quintiles • Only among **girls** with a **BMI in the 5th quintile**, **protein** intake was **inversely associated** with **changes in FMI** (ß ± SE, -0.03 ± 0.01; p for trend=0.01) • Among **boys**, **no associations** were found between protein intake and changes in **FMI** • **No associations** were found between **protein** intake and **FFMI** among girls or boys

Table 4 *continued.*

Author	Design and population	Exposure and Outcome	Results
van Vught et al 2010 *continued*		**Outcome:** 3 year change in body composition: FFMI and FMI (estimated using skinfold measurements; measured at baseline and follow up) **Covariates:** Baseline FMI or FFMI, age, energy intake, physical activity, and socio-economic status	
Berkey et al 2005 [299] US	• Data from a prospective cohort study, (Growing Up Today Study); started in 1996 • n=12,829 (5,550 males) with complete baseline (9-14 years of age) and 3 year follow-up (12-17 years of age)	**Exposure:** Past year milk intake (daily servings) **Mean daily milk intake** at baseline was 2.2 and 1.9 servings among boys and girls, respectively Among **children who completed the FFQ all 4 years**, boys consumed on average 2.3 servings milk per day in 1996 but only 2.0 by 1999, and girls consumed 2.0 servings in 1996, which declined to 1.7 by 1999 **Assessment method:** Semi-quantitative 132 item FFQ (typical past-year intake) **Outcome:** BMI change per year (year following the FFQ; self-reported heights and weights) **Covariates:** Race/ethnicity, age, same-year height growth, prior BMI z-score, pubertal stage, menstrual history, and same-year physical activity and inactivity, energy intake	• **Continuous milk** intakes were **related** to small **BMI increases per year** (ß ± SE, 0.019 ± 0.009 kg/m^2; p for trend=0.03 and 0.015 ± 0.007 kg/m^2 per serving a day; p for trend=0.04, for males and females respectively) • **No relations** were seen between **whole milk or 2% milk** and change in BMI per year (p for trend>0.1) • **Skim milk** (females) and **1% milk** (males) were **associated with BMI gain** per year (ß=0.020 kg/m^2 and ß=0.027 kg/m^2 per serving a day, respectively; both p for trend<0.05), however, among girls the relation attenuated after adjusting for energy intake (ß=0.020 kg/m^2 per serving a day; p for trend=0.09) • **Total energy** intake was the most **important predictor of weight gain** (multivariate analyses)
Koppes et al 2009 [300] The Netherlands	• Data from a prospective cohort study (Amsterdam Growth and Health Longitudinal Study); started in 1977; prospective and cross-sectional analyses	**Exposure:** Dietary protein (%E) (measured at 13, 14, 15, 16, 22, 28, 32, and 36 years of age) **Mean protein intakes were not reported** **Assessment method:** Cross-check dietary history interview	• Dietary **protein** intake at age 13 was **not related** to the sum of the four **skinfolds** at 36 years • Among **women with body fat >35% at the age of 36 years**, dietary **protein** intake was about **1% higher at the age of 13** (p for difference<0.001), 32 and 36

Table 4 *continued.*

Author	Design and population	Exposure and Outcome	Results
Koppes et al 2009 *continued*	• n=350 (168 males) with complete baseline and follow up data • Mean age (SD) at baseline: 13 (0.7) years of age • 23 year follow-up	**Outcome:** Sum of skinfolds (measured at baseline and after 1, 2, 3, 8, and 19), %BF (DEXA; used at the last measurement, 23 year follow-up), and energy intake **Covariates:** Age, level of education (at 36 years of age) and smoking; analyses with total energy intake and sum of skinfolds are additionally adjusted for the physical activity level; energy intake is considered to be a mediator	years (for both p for difference<0.01) than in women with lower %BF; no differences were found at other time points (14, 15, 16, 22 or 28 years of age) • Among **men with body fat >25% at the age of 36 years**, dietary **protein** intake was about **1% higher at the ages of 32 and 36 years** (p<0.05 and p<0.01, respectively) than compared to men with lower %BF; **no differences were found at other time points** (13, 14, 15, 16, 22 or 28 years of age)

[1] Only relevant exposures and outcomes are presented; Adjusted p-values are presented, if not indicated otherwise

Clinical trials

Six randomised controlled trials were identified examining the impact of milk, dairy and/or meat interventions and body composition and weight loss among children and adolescents. One included a 1 year intervention and was conducted the US [301], the second involved a 1.5 year intervention and was conducted in the UK [30]. The next two randomised controlled trials both included a 2 year intervention period and were conducted in New Zealand [302] and Kenya [303]. The last two studies were with 7 days and 16 weeks only rather short-term interventions, conducted in Denmark [29] and Chile [304], respectively (**Table 5**).

A 1.5 year intervention study did not show an effect of the milk intervention on weight, fat mass, and lean muscle mass among 82 adolescents aged 12 years [30] and in another 2 year intervention study dairy products had no impact on weight, fat mass, and lean muscle mass among 73 adolescents aged 15-16 years [302]. Similarly, another study did not find an impact of a 1 year intervention of dairy products on weight, body fat, and fat-free mass among 11 year old adolescents (n=46) [301]. These three studies were originally designed to investigate changes in bone mineral composition [30, 301, 302], only one specifically included body composition in the aim [301], and had smaller sample sizes (n<90).

A 7 day intervention did not show differences in weight or BMI between a milk and meat supplementation among 24 boys aged 8 years. However, milk supplementation resulted in weight gain and thus an increase in BMI, while there were no changes in the meat group [29]. Among 98 Chilean girls, who were counselled to drink 3 portions of the milk beverages per day and not to consume sugar sweetened beverages, a 16 week intervention resulted in an additional gain in fat-free mass, but not weight, BMI, or fat mass, in comparison with the control group [304]. A larger study including 910 Kenyan children aged 6-14 years showed an effect of meat as well as milk supplementation over 2 years on the mid-upper-arm muscle area, but not mid-upper-arm fat area [303].

Taken together, data from clinical trials among children and adolescents either suggest no effect of dietary protein intake in form of milk or dairy products on body composition or they suggest that dietary protein intake in form of milk and meat could lead to a higher lean body mass.

Table 5 Clinical trials in children and adolescents assessing the relation between dietary protein intake, weight loss and adiposity measures[1]

Author	Design and population	Exposure and Outcome	Results

Cadogan et al 1997 [30] UK	• Randomised controlled trial; start not reported
	• White girls randomised to either 568 mL of whole or reduced fat milk per day for 1.5 years (n=44) or no intervention (n=38)
	• Mean age (SD): 12.2 (0.3) years
		Randomisation groups:
		Milk group, i.e. whole or reduced fat milk with the same Calcium content
		  The daily **mean (SD) dietary protein intake** was 59.1g (14.2) accounting for 12.5%En at baseline and 70.3g (13.6) accounting for 14.0%En at 1.5 years
		Control group, no milk, girls continued with their habitual diet
		  The daily **mean (SD) dietary protein intake** was 55.8g (11.7) accounting for 11.9%En at baseline and 56.4g (9.9) accounting for 12.8%En at 1.5 years
		  (%En was estimated from table 2)
		Assessment method: 7-day weighed food record (assessed at baseline and end of study) and 4-day unweighed food diary (five interim occasions)
		Outcome: Weight, fat mass, %BF, and lean body mass (measured at baseline, 6, 12, and 18 months)
		Covariates: pubertal status
			• **No differences between the groups**, both groups showed similar increases in weight, fat mass, and lean body mass (no p-values reported)
			• The **milk group** showed **a tendency towards greater gain in weight** (+8.0 kg vs. +7.2 kg) and lean body mass (+5.6 kg vs. +5.1 kg), and **reduction in %BF** (-1.4% vs. +0.4%) compared to the control group, albeit non-significant; no p-values given (absolute data approximated from graph showing %changes)

Merrilees et al 2000 [302] New Zealand	• Randomised controlled trial; started in 1993
	• Healthy girls (n=105) randomised to either a 2 year dairy intervention (dairy food products (≥1000 mg/d), including milk, flavoured milk, dairy dessert, cheese or yoghurt; low fat options were available; supplements were provided fortnightly) or no intervention
		Randomisation groups:
		Dairy group
		  At baseline, 2, and 3 years, **mean (SEM) dietary protein intake** was 62.5g (3.5), 81.2g (4.1), and 64.7g (3.3), respectively (accounting for 13.1%En, 16.0%En, and 14.6%En, respectively)
		Control group
		  At baseline, 2, and 3 years, **mean (SEM) dietary protein intake** was 66.2g (3.5), 62.4g
			• **No differences** in changes in weight, fat mass, and lean body mass (from baseline to 2 or 3 years) were observed **between the groups**

Table 5 *continued.*

Author	Design and population	Exposure and Outcome	Results
Merrilees et al 2000 *continued*	• 15-16 years of age at baseline • n=91 completed the 2 year visit • n=73 had complete data of the 3 year follow-up	(3.5), and 64.4g (2.6), respectively (accounting for 14.8%En, 14.4%En, and 14.9%En, respectively) At year 2 protein intakes differed between groups (p for difference<0.001) (%En was estimated from table 2) **Assessment method:** 3-day dietary record **Outcome:** Weight, fat mass, and lean muscle mass (DEXA) (measured at baseline, 2 and 3 years) **Covariates:** n/a	

Chan et al 1995 [301]
US
	• Randomised controlled trial with a duration of 1 year; start not reported
	• n=48 girls randomised to either a supplementation of dairy products or the usual diet
	• Mean age (SD) at baseline: 11 (1) years
	• n=46 completed the study and were included in the final analysis
		Randomisation groups:
		Dairy group, ≥1200 mg Calcium/d through milk, cheese, yoghurt
		  The daily **mean (SD) dietary protein intake** was 70g (16), accounting for 19.1%En
		Control group, usual diet continued
		  The daily **mean (SD) dietary protein intake** was 52g (16), accounting for 14.8%En
		(%En was estimated from table 2)
		Assessment method: 3-day dietary history and FFQ at baseline, after 3, 6, 9, and 12 months
		Outcome: Weight, body fat and lean body mass (DEXA) (measured at baseline, after 3, 6, 9, and 12 months)
		Covariates: n/a
			• There were **no differences between the diet groups** in lean body mass or %BF
			• Weight, body fat, and fat-free mass increased in the control group (7.2 kg, 2.6 kg, and 3.8 kg, respectively) as well as in the dairy group (6.4 kg, 2.6 kg, and 4.3 kg, respectively); no p-values given
			• The dairy group reported a significantly higher mean intake (SD) of protein (70 (16) g/d vs. 52 (16 g/d; p for difference=0.0003) compared to the control group, the total energy and fat intake did not differ between groups

Hoppe et al 2004 [29] Denmark	• Randomised controlled trial • Boys born between 10-12/1992, drawn at random from the Central Personal Register	Randomisation groups: **Milk group** At baseline and day 7, **daily mean (SD) protein intakes** were 68.6g (10.0) and 121.4g (17.2),	• **No differences in weight or BMI were observed between the groups** • The milk group **gained** an average of 550 g of weight during the intervention, causing an increase in BMI

Table 5 *continued.*

Author	Design and population	Exposure and Outcome	Results
Hoppe et al 2004 *continued*	• Randomisation to either 1.5 L low fat milk per day (n=12) or 250 g low fat meat per day (n=12) supplementation for 7 days (that is 53 g protein daily) • 8 years of age	respectively (accounting for 13.1%En and 20.6%En, respectively **Meat group** At baseline and day 7, **daily mean (SD) protein intakes** were 65.3g (11.4) and 105.6g (33.8), respectively (accounting for 12.7%En and 19.9%En, respectively Intakes at day 7 were all significantly different from intakes a baseline (all p for difference<0.001) **Outcome:** Weight and BMI (measured at baseline and day 7) **Covariates:** n/a	from 17.2 to 17.5 kg/m^2 (p for difference=0.015), while there were no changes in the meat group (29.0 and 29.0 kg, respectively)
Albala et al 2008 [304] Chile	• Randomised controlled trial; data collected between 07/2004 and 12/2005 • Overweight and obese children randomly assigned to either a 16 week milk (replacement of sugar-sweetened beverages by 3 × 200g milk/d; n=50 (26 males)) or no intervention (n=28 (26 males)) • n=47 (23 males) in the milk group and n=46 (26 males) in the control group completed the study • 8-10 years of age	Randomisation groups: **Milk group** At baseline, **daily mean (SEM) protein intake** was 87.1g (2.5) accounting for 13.2%En Change between baseline and 16 weeks was 11.5g (1.8) **Control group** At baseline, **daily mean (SEM) protein intake** was 85.5g (2.1) accounting for 13.3%En Change between baseline and 16 weeks was 1.8g (0.7) Changes in protein intake differed between groups (p for difference<0.0001) (%En was estimated from table 2) **Assessment method:** FFQ at baseline and 16 weeks	• The mean accretion of **lean body mass was greater in the intervention than in the control group** (mean (SE) 0.92 (0.10) kg vs. 0.62 (0.11) kg; p for difference=0.04) • **No differences** were observed regarding changes of **weight, BMI, total body fat, %BF or trunk body fat**

Table 5 *continued.*

Author	Design and population	Exposure and Outcome	Results
Albala et al 2008 *continued*		**Outcome:** Weight, BMI, total body fat, %BF, trunk fat, and lean mass (DEXA) (measured at baseline and 16 weeks) **Covariates:** Age, sex, and group duration sex interaction term	

Neumann et al 2013 [303] Kenya	• Randomised, controlled trial, undertaken in two cohorts of 518 and 392 schoolchildren, respectively; cohort one started between 07/1998 and 08/1998 and cohort two started between 07/1999 and 08/1999
	• Twelve elementary schools were randomly assigned to either an isoenergetic feeding interventions containing meat, milk or plain traditional vegetable stew (githeri) or a control group receiving no snack
	• 2 year intervention
	• 6-14 years of age (median age 7.4 years)
		Randomisation groups:
		Meat group
		   At baseline, **daily mean (SD) protein intake** was 57.1g (26.7) accounting for 13.5%En
		Milk group
		   At baseline, **daily mean (SD) protein intake** was 49.8g (18.6) accounting for 12.4%En
		Githeri group
		   At baseline, **daily mean (SD) protein intake** was 59.1g (32.9) accounting for 13.1%En
		Control group
		   At baseline, **daily mean (SD) protein intake** was 51.4g (20.3) accounting for 12.5%En
		   (%En was estimated from table 2)
		   For the intervention period, no intake values reported.
		Assessment method: 24 h recall every 1-2 months
		Outcomes: Weight, triceps skinfold, and mid-upper-arm circumference (MUAC); mid-upper-arm muscle area (MAMA) and mid-upper-arm fat area (MAFA) were calculated
		Covariates: Time, intervention × time, socio-economic status, age at baseline, sex, time^2, socio-economic status × time, sex × time, and school
			• The **meat group** showed the **steepest gain in MUAC and MAMA** over time (ß=0.025 and ß= 6.571, respectively) compared with the slopes of githeri and control group (MUAC: p for difference=0.0005 and 0.0001, respectively; MAMA: both p for difference<0.0001)
			• The **milk group** showed the **next largest MUAC and MAMA gain** (ß=0.018 and ß= 4.354, respectively) compared with the slopes of githeri and control group (albeit non-significant for MUAC: both p for difference>0.1; tendency for MAMA: p for difference=0.008 and 0.076, respectively)
			• **Triceps skinfold and MAFA** slopes of the three feeding groups (meat, milk and githeri) **did not differ from the control group**
			• However, the meat group showed the least increase in triceps skinfold and MAFA of all groups, even though non-significant
			• Weight increased in all three feeding groups (meat, milk and githeri) compared to control group (F-Test: p for difference=0.008)

[1] Only relevant exposures and outcomes are presented; Adjusted p-values are presented, if not indicated otherwise

2.3.3 Protein intake and GH-IGF axis

The relevance of dietary protein intake for the GH-IGF axis has not been fully elucidated yet. Cross-sectional studies in adults suggest that high (animal) protein intakes are related to higher IGF-I [305-309] and IGFBP-3 levels [308]. Moreover, sources of animal protein have been found to be an important determinant of IGF-I levels, with most studies pointing to dairy products or milk [307-309] and others to meat [305, 306]. Because the IGF system has a central role in the regulation of foetal and childhood growth and metabolism [26], protein intake in infancy and childhood in particular may be related to the GH-IGF axis (see also chapter 2.2.2). Five observational studies and seven clinical trials were identified, which examined the relation between dietary protein intake (in infancy and childhood) and the GH-IGF axis in infancy, childhood, adolescence, and adulthood (IGF-I, IGFBP-2, and IGFBP-3) (**Table 6** and **Table 7**).

Observational studies

Out of five observational studies, seven analyses were identified examining the relation between dietary protein intake and/or dairy products with the GH-IGF axis. Five analyses had a cross-sectional design including three analyses of the Avon Longitudinal Study of Parents and Children (ALSPAC) conducted in the UK [310-312]. These three analyses used ALSPAC data to examine protein intake and GH-IGF axis, albeit with slightly different sample sizes and focus (dietary total, animal, vegetable protein intake [310, 312], cow's milk and dairy products [311, 312] and meat products [310]). The other two analyses came from studies conducted in Denmark, i.e. a follow-up of a randomised intervention study examining the effect of fish oil or olive oil supplementation of lactating Danish mothers [313] and a pooled analysis of a randomised controlled trial examining whole milk and infant formula [314]. In addition, two analyses had a prospective design of which one was from a prospective cohort study conducted in the UK [32] and the other one a prospective long-term follow-up of a randomized controlled trial examining prenatal and postnatal milk supplementation [31].

Among 83 infants, positive correlations between protein intake and IGF-I at 9 and 12 months of age as well as between whole milk and whole milk products and IGF-I at 9 months of age were observed [314]. Similarly, a direct association of animal protein and milk intake, but not vegetable protein or meat intake with IGF-I were found among 90 infants aged 32 months [313]. The three analyses of ALSPAC included 7-8 year old children (n≈500 each) observing positive relations of total protein, animal protein, but not vegetable protein intake with IGF-I [310, 312]. No associations were observed for total protein, animal protein or vegetable

protein and IGFBP-3 [310, 312]. Furthermore, Martin et al also examined cow's milk and dairy intake which was positively associated with IGFBP-3, but not IGF-I [312] while Rogers et al investigated dietary meat intake which was not related to IGF-I or IGFBP-3 [310]. The third ALSPAC analysis, published one year later, focussed on cow's milk and dairy products only, finding a direct relation with IGF-I and also IGFBP-3. Furthermore, sex-stratified results were presented showing relations between cow's milk and dairy products and IGF-I and IGFBP-3 only among boys [311].

Only two analyses had a prospective design, addressing the long-term relevance of (animal) protein intake and/or its sources during growth in relation to the GH-IGF axis. No relations between protein or meat intakes in early childhood and IGF-I in older age (n=679; 65 years of follow-up) were found [32], but both studies observed an inverse association between milk intakes in early childhood and IGF-I levels in young adulthood (n=352; 25 years of follow-up) [31] and in older age [32]. The authors proposed that this inverse relation reflects an early programming of the GH-IGF axis in response to higher (animal) protein intakes in early life. Under this hypothesis, an early pituitary resetting in response to higher ambient IGF-I concentrations may occur, which would ultimately result in an inverse association between animal protein intake in early life and IGF-I levels in young adulthood. No associations were observed with regards to IGFBP-2 or IGFBP-3.

In conclusion, cross-sectional studies indicate a direct relation between total protein, animal protein and/or dairy protein intake with IGF-I in infancy and childhood. By contrast, prospective studies rather suggest an inverse relation between milk intakes and IGF-I in later life. With regards to IGFBP-3, observational studies do not support a strong relation with protein intakes. Only two cross-sectional studies and one prospective study have examined meat intake and the GH-IGF axis and do not support an association between them.

Table 6 Observational studies in infants and children assessing the relation between dietary protein intake and GH-IGF axis[1]

Author	Design and population	Exposure and Outcome	Results
Larnkjær et al 2009 [314] Denmark	• Cross-sectional • Pooled analysis within an randomised controlled trial; started in 2003 • Healthy term infants randomised to either whole milk (n=38 (16 males)) or infant formula (IF, n=45 (25 males)) and either a daily fish oil supplement (FO) or no supplement (2x2 factorial design) • n=83 (41 males)	**Exposure:** Dietary protein (%En, at 9 and 12 month of age) **Daily mean dietary intake among the pooled sample was not reported** **Assessment method:** 7-day precoded dietary record **Outcome:** IGF-I (at 9 and 12 months of age) **Covariates:** Sex and duration of full breastfeeding	**At 9 months of age:** • **Positive correlation** between protein intake (%En) and IGF-I (r=0.329; p=0.015) • **Positive correlation** between intake of whole milk and whole milk products and IGF-I (r=0.356; p=0.008) **At 12 months of age:** • **Positive correlation** between protein intake (%En) and IGF-I (r=0.272; p=0.044)
Hoppe et al 2004 [313] Denmark	• Cross-sectional • Data from a follow-up examination of an intervention study [315]; carried out from 11/2001 to 09/2002 • n=90 (54 males) healthy term singletons with complete blood data at follow-up • Mean age (SD) of girls and boys at follow-up was 31.9 (0.86) months and 31.6 (0.89) months, respectively	**Exposure:** Dietary total, animal, and vegetable protein intake (g), milk (g), and meat intake (g) Among boys, **daily mean (SD) dietary total, animal and vegetable protein intakes** were 41.8g (8.3), 26.2g (6.4), and 14.5g (4.2), respectively; **daily milk and meat intakes** were 369g (130) and 39.4g (25.9), respectively Among girls, **daily mean (SD) dietary total, animal and vegetable protein intakes** were 43.5g (10.8), 28.4g (7.8), and 13.9g (4.1), respectively; **daily milk and meat intakes** were 410g (179) and 33.7g (16.3), respectively **Assessment method:** 7 day dietary questionnaire (adapted from Danish National Food Survey) **Outcome:** Serum IGF-I **Covariates:** Sex, weight, previous meal size, birth size (weight and length), and parental height	• **Positive correlation** between total protein, animal protein or milk intake and IGF-I (p<0.05) • Multiple linear regression analysis showed **direct relations** of **animal protein and milk intake** with **IGF-I** (ß ± SE 1.4 ± 0.53ng/mL; p for trend=0.013 and 0.049 ± 0.024ng/mL; p for trend=0.045, respectively) • **Vegetable protein and meat intake** were **not related** to **IGF-I**

Table 6 *continued.*

Author	Design and population	Exposure and Outcome	Results
Rogers et al 2005 [310] UK	• Cross-sectional • Data from a prospective birth cohort; started between 04/1991 and 12/1992 (**ALSPAC**) • The children forming the basis of this analysis were part of a randomly selected 10% sub-cohort of ALSPAC called Children in Focus (n=1,335) • n=521 (287 males) children aged 7-8 years with complete blood data at follow-up	**Exposure:** Dietary total, animal, and vegetable protein intake (g), red meat (g), processed meat (g), and poultry (g) Among boys, **daily mean (IQR) dietary total, animal and vegetable protein intakes** were 56.3g (49.0, 65.4), 34.5g (26.7, 40.7) and 22.2g (18.8, 26.5), respectively; **daily red meat, processed meat, and poultry intakes** were 38g (20, 73), 32g (15, 53) and 28g (0, 47), respectively Among girls, **daily mean (IQR) dietary total, animal and vegetable protein intakes** were 52.0g (46.5, 60.1), 30.6g (25.0, 37.6), and 21.8g (17.9, 25.3), respectively; **daily red meat, processed meat, and poultry intakes** were 40g (19, 70), 30g (8, 55), and 20g (0, 38), respectively **Assessment method:** 3-day unweighed dietary record (1week before clinic) **Outcome:** Serum IGF-I, IGFBP-3, and IGF-I/IGFBP-3 ratio **Covariates:** Energy intake, sex, maternal education, housing tenure, birth weight, and BMI	• **Total and animal protein, but not vegetable protein** intakes **were positively correlated** with **IGF-I** (r=0.19 and r=0.16; both p<0.001, respectively) and the **IGF-I/IGFBP-3 ratio** (r=0.14; p<0.002 and r=0.14; p<0.003, respectively), adjusted for age, sex, and energy intake • **No correlation** between **total, animal or vegetable protein** and **IGFBP-3** • **Red meat, processed meat or poultry** were **not related** to **IGF-I, IGFBP-3** or the **IGF-I/IGFBP-3 ratio**
Martin et al 2005 [312] UK	• Cross-sectional • Data from a prospective birth cohort; started between 04/1991 and 12/1992 (**ALSPAC**) • The children forming the basis of this analysis were part of a randomly selected 10% sub-cohort of ALSPAC called Children in	**Exposure:** Dietary total, animal, and vegetable protein intake (g), cow's milk (g), and dairy products (g) The **daily mean (SD) total, animal, and vegetable protein intakes** were 55.6g (12.4), 32.9g (11.3), and 22.7g (6.0), respectively, for participants who had been breastfed and 55.1g (13.9), 34.2g (13.4), and 20.8g (4.8), respectively, for participants who	• **Total protein and animal protein** intakes, **but not vegetable protein**, were **positively associated** with **IGF-I** (median (IQR): 0.8 (0.4, 1.3); p for trend<0.001 and 0.6 (0.2,1.0); p for trend<0.01, respectively; values are change in IGF-I per unit increase in continuous variable) • **No associations** between **total, animal or vegetable protein** and **IGFBP-3**

Table 6 *continued.*

Author	Design and population	Exposure and Outcome	Results
Martin et al 2005 *continued*	Focus (n=1,215) n=488 (267 males) children aged 7-8 years with complete blood data at follow-up	had never been breastfed The **daily median (IQR) intakes for cow's milk and dairy products** were 233g (110, 369) and 300g (186, 461), respectively, for participants who had been breastfed and 240g (112, 340) and 292 (184, 445), respectively, for participants who had never been breastfed **Assessment method:** 3-day unweighed dietary record (1week before clinic) **Outcome:** Serum IGF-I and IGFBP-3 (measured at follow-up) **Covariates:** Current age, sex, energy intake using the residuals method	• **Cow's milk and dairy** intake were **positively associated** with **IGFBP-3** (median (IQR): 119.5 (−0.4, 239.5); p for trend=0.05 and 139.6 (17.9, 261.2); p for trend=0.05, respectively; values are change in IGFBP-3 per unit increase in continuous variable), **but not IGF-I**
Rogers et al 2006 [311] UK	• Cross-sectional • Data from a prospective birth cohort; started between 04/1991 and 12/1992 (**ALSPAC**) • The children forming the basis of this analysis were part of a randomly selected 10% sub-cohort of ALSPAC called Children in Focus (n=1,432) • n=538 (295 males) children aged 7-8 years with complete blood data at follow-up	**Exposure:** Cow's milk, dairy products (g) Among boys, **daily mean (SD) cow's milk and dairy product intakes** were 278g (195) and 348g (209), respectively Among girls, **daily mean (SD) cow's milk and dairy product intakes** were 243g (187) and 313g (193), respectively **Assessment method:** 3-day unweighed dietary record (1week before clinic) **Outcome:** Serum IGF-I and IGFBP-3 **Covariates:** Energy intake, sex, maternal education, housing tenure, birth weight, and BMI	• In the total sample, **cow's milk and dairy product** intakes were **positively associated** with **IGF-I** (p for trend=0.040 and 0.027, respectively) and **tended to be associated** with **IGFBP-3** levels (p for trend=0.082 and 0.067, respectively) • After sex-stratification, both **cow's milk and dairy product** intakes were **positively associated** with **IGF-I** (p for trend=0.084 and 0.031, respectively) and **IGFBP-3** levels (p for trend=0.024 and 0.022, respectively) among **boys** • **No relations** were seen among **girls**

Table 6 *continued.*

Author	Design and population	Exposure and Outcome	Results
Long-term follow-up			
Martin et al 2007 [32] UK	• Prospective birth cohort (Carnegie (Boyd Orr Cohort) Survey of Diet and Health in Pre-War Britain); started between 1937 and 1939 • Median age at baseline: 5.8 (IQR: 2.9, 9.6) • n=679 with complete data • Mean age at follow-up: 71.1 years (range 64.0-82.6)	**Exposure:** Dietary protein intake (g), milk and milk product (g), and meat intake (g) at baseline Per person, **the daily mean (SD) childhood households dietary protein, milk and milk product, and meat intake** was 65g (16), 258g (188), and 85g (38), respectively **Assessment method:** 7-day household food inventories **Outcome:** Serum IGF-I, IGFBP-2, and IGFBP-3 (measured at follow-up) **Covariates:** Adult age, sex, type of sample (clinic or bloods by post), energy intake, social class in childhood, social class in adulthood, and lifestyle factors (pack-years of smoking, alcohol consumption, levels of exercise and BMI); IGF-I additionally adjusted for IGFBP-3; IGFBP-3 adjusted for IGF-I	• **Milk and milk product** intake during childhood was **inversely associated** with **IGF-I** in older age (change in IGF-I -2.5 (95% CI -5.1, -0.1) ng/mL; p for trend=0.05); fully adjusted model • **No associations** between **protein or meat** intake during childhood and **IGF-I** in older age • **No associations** between **protein, milk and milk product or meat** intake during childhood and **IGFBP-2 or IGFBP-3** in older age
Ben-Shlomo et al 2005 [31] South Wales	• Long-term follow-up of a randomised controlled trial of prenatal and postnatal milk supplementation; 1972-1974 • Pregnant women were randomised to either the milk intervention or control group (no milk supplementation) Milk tokens were provided throughout pregnancy and subsequently for their child until the until the age of 5 years	Randomisation groups: **Milk group** **Control group** **Mean milk or protein intakes were not reported** **Outcome:** Serum IGF-I, IGFBP-3, molar IGF-I/IGFBP-3 ratio (measured at 25 year follow up) **Covariates:** Adult age, sex, maternal systolic blood pressure, maternal smoking, birth weight, birth length, gestational age, adult smoking behaviours, alcohol consumption, and adult BMI	• Subjects in the **milk group in prenatal/early life** period had **lower adult IGF-I** levels (-8.5 (95% CI -15.1, -1.8) ng/mL; p for difference=0.01) and **lower** adult molar **IGF-I/IGFBP-3 ratio** (-1.20 (95% CI -2.33, -0.04); p for difference=0.04) **compared to the control group** • Differences could not be explained by follow-up bias or confounding factors • **No differences** between the groups were seen for **IGFBP-3**

Table 6 *continued.*

Author	Design and population	Exposure and Outcome	Results
Ben-Shlomo et al 2005 *continued*	• Children with complete blood data at 25 year follow-up (1997-1999) were included (milk group n=352, control group n=312) • Mean age at follow-up: 25 years		

[1] Only relevant exposures and outcomes are presented; Adjusted p-values are presented, if not indicated otherwise

Clinical trials

Seven intervention studies were identified that examined dietary protein intake and the GH-IGF axis. Of these, two studies were conducted in infants, of which one randomised trial examined whole milk vs. regular infant formula (over a 3 months intervention period) in Denmark [314], the other multicentre, double-blind, randomised controlled trial, i.e. the EU CHOP, studied cow's milk formulas with different protein content (over the first year of life). It should be noted that pooled results regarding the GH-IGF axis were published twice, albeit with slightly different sample sizes and objectives [256, 316]; Closa-Monasterolo et al also examined whether sex modified the GH-IGF axis in response to protein intakes in early life [316]. In addition, five studies were conducted in children. A Danish randomised controlled trial evaluated a 7 day milk vs. meat intervention [29]. The other three randomised controlled trials studied a milk interventions vs. a control in the UK (18 months duration) [30], in China (24 months duration) [28], and the US (2 × 2 weeks cross-over design) [27]. The last study was a 1 months pilot study which studied the influence of a milk intervention in Mongolia [27].

Analyses of CHOP included 577 and 584 infants, respectively [256, 316], results showed that high protein cow's milk formula intake in early life increased IGF-I and decreased IGFBP-2 levels at 6 months of age compared to low protein cow's milk formula [256, 316]. Compared to infant formula, no overall effect of a 3 months whole milk intervention, starting at 9 months of age, on IGF-I levels at the age of 12 months was observed (n=83), whereas an effect of whole milk on increased IGF-I levels was seen among boys only [314]. By contrast, Closa-Monasterolo et al observed that only among female infants high protein cow's milk formula increased IGF-I levels at 6 months of age compared to low protein cow's milk formula. Furthermore, their sex stratified analysis revealed that decreases in IGFBP-2 levels due to high protein cow's milk formula were of a greater magnitude in girls compared to boys (410.8 ng/mL vs. 271.1 ng/mL, respectively) [316]. No effect of whole milk or high protein formula on IGFBP-3, were observed [256, 314, 316].

Among 24 boys aged 8 years, milk supplementation over 7 days increased IGF-I and IGFBP-3 levels and the IGFI-I/IGFBP-3 ratio at day 7 compared to baseline, while no differences were seen among the meat group. No differences were observed between the groups [29]. An 18 months milk intervention resulted in higher IGF-I concentrations among 82 girls ages 12 years [30] and a 24 months school-milk intervention increased IGF-I levels at the endpoint among 10 year olds compared to the control group [28]. Similarly, a 2 week milk

supplementation led to small, but non-significant increases in IGF-I concentrations and the IGF-I/IGFBP-3 ratio among 28 pre-pubertal girls [27]. The 1 months pilot study among 46 children aged 10-11 years showed increases in in IGF-I levels and the IGF-I/IGFBP-3 ratio compared to baseline [27].

Hence overall, the majority of the presented clinical trials support an effect of high protein intakes, consumed as cow's milk, to increase IGF-I and decrease IGFBP-2 levels during infancy and childhood, but yet available data does not strongly support an influence on IGFBP-3 concentrations. Only one study has examined meat intake and did not find any influences on the GH-IGF axis.

Table 7 Clinical trials in infants and children assessing the relation between dietary protein intake and GH-IGF axis[1]

Author	Design and population	Exposure and Outcome	Results
Infants			
Socha et al 2011 [256] Europe: Germany, Belgium, Italy, Poland, and Spain	• Multi-centre, double-blind randomised clinical trial; CHOP started between 10/2002 and 07/2004 • Healthy infants randomised to either a lower (n=291) or higher protein cow's milk-based infant formula (first 8 weeks of life) and follow-on formula until the age of 12 months (n=286) and an observational group of breastfed infants (Control group: n=187) • Median age at study entry: 14 days • Complete blood data at the age of 6 months	Randomisation groups: **Low protein group** (LP) 1.77 and 2.2 g/100 kcal, respectively **High protein group** (HP) 2.9 and 4.4 g/100 kcal, respectively **Mean protein intakes were not reported** **Control group**, breastfed infants **Outcome:** Serum total IGF-I, free IGF-I, IGFBP-2, and IGFBP-3 (measured at 6 months of age) **Covariates:** Baseline measurement of weight-for-length	• **Total and free IGF-I** were about 40% **higher** in the **HP group compared to the LP group** (median total IGF-I 48.4 (IQR: 27.2, 81.8) vs. 34.7 (IR: 17.7, 57.5) ng/mL; p for difference<0.001) • **IGFBP-2** was about 30% **lower in the HP group compared to the LP group** (765 (IR: 575,1013) vs. 1090 (IR: 865, 1438) ng/mL; p for difference<0.001) • **IGFBP-3 did not differ between groups**

Closa-Monasterolo et al 2011 [316] Europe: Germany, Belgium, Italy, Poland, and Spain	• Multi-centre, double-blind randomised clinical trial; CHOP started between 10/2002 and 07/2004
	• Healthy infants randomised to either a lower (n=290 (137 males)) or higher protein cow's milk-based infant formula (first 8 weeks of life) and follow-on formula until the age of 12 months (n=294 (149 males)) and complete blood data at the age of 6 months
	• Observational group of breastfed infants (Control group) was not
		Randomisation groups:
		Low protein group (LP)
		1.77 and 2.2 g/100 kcal, respectively
		  At 3 months, the **median daily protein intake** was 1.7g/kg (approximated from figure 2)
		  At 6 months, the d**aily median (IQR) protein intake** was 1.98g/kg (1.70, 2.31)
		High protein group (HP)
		2.9 and 4.4 g/100 kcal, respectively
		  At 3 months, the **median daily protein intake** was 2.7g/kg (approximated from figure 2)
		  At 6 months, the **daily median (IQR) protein intake** was 3.11g/kg (2.59, 3.57)
			• **HP formula** was associated with **higher** concentrations of **free IGF-I** and **lower** concentrations of **IGFBP-2 compared to the LP formula** (p for difference<0.001 for all cases)
			• **No differences between HP and LP formula regarding IGFBP-3**
			• Interaction between sex and formula for IGFBP-2 (p for interaction=0.04) and total IGF-I (p for interaction=0.06)
			• In **female infants HP formula** induced an **increase** in **total IGF-I of** 24.4 ng/mL (95% CI 11.4, 37.3; p for difference<0.001) **compared to LP formula**, which was **not seen among male infants** (mean difference was 11.2 ng/mL (95% CI 1.9, 24.3; p for

Table 7 *continued.*

Author	Design and population	Exposure and Outcome	Results
Closa-Monasterolo et al 2011 *continued*	included in this study	Overall, intakes were not different between boys and girls **Outcome:** Serum total IGF-I, free IGF-I, IGFBP-2, and IGFBP-3 (measured at 6 months of age) **Covariates:** Weight	difference=0.1)) • **HP formula** induced an **increase** in **IGFBP-2** of 410.8 ng/mL (95% CI 283.7, 538.0; p for difference<0.001) among female infants and 271.1 ng/mL (95% CI 142.4, 399.8; p for difference<0.001) among male infants **compared to LP formula** • Compared to male infants, female infants showed higher concentrations of total and free IGF-I and IGFBP-3 at 6 months of age (p= 0.002; p= 0.04, and p for difference<0.001, respectively)
Larnkjær et al 2009 [314] Denmark	• Randomised clinical trial; started in 2003 • Healthy term infants randomised to either whole milk (n=38 (16 males)) or infant formula (n=45 (25 males)) and either a daily fish oil supplement (FO) or no supplement (2x2 factorial design); 3 months intervention • Those still breastfed continued to do so	Randomisation groups: **Whole milk** plus FO At 9 and 12 months, the **daily mean (SD) dietary protein intake** was 11.8%En (1.7) and 11.4%En (1.3), respectively Any **infant formula** with protein content between 1.1 and 1.5 g protein/100 mL, no FO At 9 and 12 months, the **daily mean (SD) dietary protein intake** was 11.8%En (2.5) and 14.2%En (2.2), respectively **Outcome:** Weight, serum IGF-I, and IGFBP-3 (measured at baseline (9 months of age) and 12 months of age) **Covariates:** Breastfeeding	• Overall, **whole milk** had **no effect on IGF-I** • **Whole milk had no effect on IGFBP-3** • Among **boys**, randomization to **whole milk increased IGF-I** by 27% **compared to the infant formula group** (p for difference≤0.05, **but not in girls** • Intake of fish oil had no effect on the outcomes

Table 7 *continued.*

Author	Design and population	Exposure and Outcome	Results
Children			
Hoppe et al 2004 [29] Denmark	• Randomised controlled trial • Boys born between 10-12/1992, drawn at random from the Central Personal Register • Randomisation to either 1.5 L low fat milk per day (n=12) or 250 g low fat meat per day (n=12) supplementation for 7 days (that is 53 g protein daily) • 8 years of age	Randomisation groups: **Milk group** At baseline and day 7, daily **mean (SD) protein intakes** were 68.6g (10.0) and 121.4g (17.2), respectively (accounting for 13.1%En and 20.6%En, respectively **Meat group** At baseline and day 7, **daily mean (SD) protein intakes** were 65.3g (11.4) and 105.6g (33.8), respectively (accounting for 12.7%En and 19.9%En, respectively Intakes at day 7 were all significantly different from intakes a baseline (all p for difference<0.001) **Outcome:** IGF-I, IGFBP-3, and IGF-I/IGFBP-3 ratio (measured at baseline and day 7) **Covariates:** n/a	• IGF-I, IGFBP-3, and IGF-I/IGFBP-3 ratio at day 7 **did not differ** between the milk and meat group • In the milk group, IGF-I **increased** by 39.7 ng/mL (i.e. 19%; p for difference<0.001), IGFBP-3 by 194 ng/mL (i.e. 5%; p for difference<0.001) and the IGF-I/IGFBP-3 ratio by 0.03 (i.e. 13%; p for difference<0.0001) from baseline to day 7 • **No changes** were seen in the meat group
Cadogan et al 1997 [30] UK	• Randomised controlled trial; start not reported • White girls randomised to either 568 mL of whole or reduced fat milk per day for 18 months (n=44) or no intervention (n=38) • Mean age (SD): 12.2 (0.3) years	Randomisation groups: **Milk group**, i.e. whole or reduced fat milk with the same Calcium content The **daily mean (SD) dietary protein intake** was 59.1g (14.2) accounting for 12.5%En at baseline and 70.3g (13.6) accounting for 14.0%En at 1.5 years **Control group**, no milk, girls continued with their habitual diet The **daily mean (SD) dietary protein intake** was 55.8g (11.7) accounting for 11.9%En at baseline and 56.4g (9.9) accounting for 12.8%En at 1.5 years	• The milk group showed **higher** concentrations of IGF-I over the course of the study compared to the control group (p for difference=0.08) which was significant after adjustment for pubertal status (mean increase: 132 (i.e. 33%) vs. 63 (i.e. 16%) ng/mL; p for trend=0.02)

Table 7 *continued.*

Author	Design and population	Exposure and Outcome	Results
Cadogan et al 1997 *continued*		(%En was estimated from table 2) **Outcome:** Serum IGF-I (measured at baseline, 6, 12, and 18 months) **Covariates:** Pubertal status	
Zhu et al 2005 [28] China	• Randomised controlled trial; start not reported • Chinese girls randomised to either daily 330 mL calcium-fortified milk (n=43), calcium and vitamin D-fortified milk (n=44) or control group (n=41) according to their schools in a 24 months school milk intervention trial with complete blood data • Mean (SD) age at baseline: 10 (0.3) years	Randomisation groups: **Ca milk group** At baseline and 2 years, the **mean (SD) daily protein intake** was 52.0g (14.3) and 54.5g (14.7), respectively **CaD milk group** At baseline and 2 years, the **mean (SD) daily protein intake** was 53.7g (15.0) and 58.1g (17.3), respectively **Control group**, no supplementary milk and consumed their usual diet At baseline and 2 years, the **mean (SD) daily protein intake** was 54.6g (14.8) and 54.4g (16.3), respectively **Outcome:** Plasma IGF-I (measured at 12 and 24 months) **Covariates:** Baseline IGF-I, pubertal status, menarcheal status at 12 and 24months, school clustering	• **Milk** supplementation had **increased IGF-I** concentrations at 24 months compared to the control group (adjusted percentage difference from baseline to 24 months: Ca milk group: 16.7% (95% CI 1.8, 31.6); p for difference=0.03; CaD milk group: 23.3% (95% CI 10.6, 36.0); p for difference=0.001). Adjustment for clustering by school attenuated effect (both p≥0.1) • In all 3 groups plasma IGF-I at 24 months was **higher** compared to baseline (p for difference<0.001)

Table 7 *continued.*

Author	Design and population	Exposure and Outcome	Results
Rich-Edwards et al 2007 [27] US	• Randomised cross-over feeding study; start not reported • 5 week protocol, including 2 intervention weeks with either 710 mL milk or milk substitute per day, and an intervening 3 week 'wash out' return to normal diet • Prepubertal girls (n=28) were randomised to the order of the interventions • 6-8 years of age	Randomisation groups: **Milk supplement**, 2% fat cow's milk per day **Control**, milk substitute with the same calorie, protein, fat, carbohydrate, Calcium, and Vitamin D content as 2% fat cow's milk (containing coconut milk, almond milk, and protein powder) Otherwise dairy-free diets Overall, **mean (SD) daily servings of milk and other dairy** were 14.9 (5.8) and 18.9 (10.3), respectively **Outcome:** GH, IGF-I, and IGF-I/IGFBP-3 ratio (measured at the end of each interventional week in the US) **Covariates:** n/a	• After a week of drinking 2% fat milk, Boston girls had **small** and **non-significant increases** in IGF-I, IGF-I/IGFBP-3 ratio and GH
Rich-Edwards et al 2007 [27] Mongolia	• Pilot study (05-06/2005): 1 month 710 mL/d milk supplementation among school children (n=46 (24 males)) • 10-11 years of age	**Milk supplement,** conventional US UHT-pasteurized vitamin D fortified whole milk The **mean (SD) daily servings of milk and other dairy** were 0.8 (1.3) and 6.5 (6.0), respectively, among boys and 0.5 (1.0) and 4.1 (4.0), respectively, among girls **Outcome:** Plasma GH, IGF-I, and IGFBP-3 (measured at baseline and after 1 months)	• After 1 month of drinking whole milk, Mongolian children had **higher** mean plasma levels of IGF-I (13.34 ng/mL, i.e. 5%; p for difference<0.0001), IGF-I/IGFBP-3 ratio (0.002, i.e. 14%; p for difference<0.0001), and 75th percentile of GH levels (0.36 ng/mL, i.e. 103%; p for difference=0.005)

[1] Only relevant exposures and outcomes are presented; Adjusted p-values are presented, if not indicated otherwise

2.4 Conclusive considerations

Taken together the results from observational and intervention studies among children and adolescents, the evidence on a potential benefit of low-GI and/or GL for the development of body composition and weight loss is still limited. With regards to mechanisms linking high-GI and GL to obesity it is not clear whether high postprandial glucose or insulin responses are involved in an unfavourable development of body composition. To date, prospective data relating dietary insulin demand during adolescence to body composition development and weight loss in obese adolescents is lacking *(Aim 1, Research question 1 and 2)*.

Current evidence relating protein intake and the development of body composition among children and adolescents raises the question whether dietary protein has differential effects on fat mass and/or fat-free mass. Research has focussed on total protein intakes as well as milk, dairy products, and meat intakes among children and adolescents. However, the relevance of animal protein – and its sources meat and dairy protein – as well as plant protein intakes in different potentially critical periods, namely early life, adiposity rebound, and puberty, is not fully established *(Aim 2, Research question 3)*.

To date, there exists controversy among observational studies whether there is a direct or inverse relation between protein intake and the GH-IGF axis. Although the majority of clinical trials support effects of higher protein intakes consumed as milk on the GH-IGF axis, data for protein consumed as meat is scarce. It remains to be elucidated whether habitual intakes of animal protein and its sources meat and dairy protein as well as plant protein have a long-term relevance for the GH-IGF axis. Prospective inverse relations of (animal) protein intakes in early life with the GH-IGF axis have been shown, which may reflect an early programming of the GH-IGF axis. Hence, prospective evidence covering different, potentially critical, developmental periods is lacking, so as to unravel whether an inverse association between animal protein intake and the GH-IGF axis is confined to early life *(Aim 2, Research question 4)*.

3. AIMS AND RESEARCH QUESTIONS

As summarized in the previous chapters, dietary insulin demand and dietary protein intake might play an important role in the development of body composition and/or weight loss. To date, evidence for the role of insulin demand and protein intake in childhood and adolescence is limited. Furthermore, protein intake might be relevant in the programming and development of the GH-IGF axis. However, evidence covering different developmental periods is lacking. To address these issues the following two aims, i.e. four research questions have been formulated:

Aim 1 To examine the dietary insulin demand, body composition and weight loss

Research question 1.1 Are dietary insulin demand, glycaemic index, and glycaemic load during puberty prospectively associated with body composition in young adulthood?

Research question 1.2 Are the dietary glycaemic load and insulin load associated with weight loss, changes in percentage of body fat and insulin sensitivity in obese adolescents with clinical features of insulin resistance?

Aim 2 To examine dietary protein sources, body composition and the GH-IGF axis

Research question 2.1 Are different dietary protein sources during childhood and adolescence prospectively related to body composition in young adulthood?

Research question 2.2 Are different dietary protein sources during childhood and adolescence prospectively associated with the growth hormone-insulin-like-growth-factor axis in younger adulthood?

This thesis aimed to address these research questions using data from the DONALD study and the RESIST study. The DONALD study entails repeated assessments of dietary intake, anthropometry, and metabolism in healthy German children from birth until adulthood (see chapter 4.1). With regards to dietary intake, data on the dietary insulin demand, GI, and GL were available for puberty, while data on dietary protein intake and its sources were available for the periods of early life, adiposity rebound, and puberty. The RESIST study included a 3 months lifestyle and metformin intervention designated to examine the efficacy of two diets differing in macronutrient content, i.e. lower vs. increased protein content. The RESIST study provided the required data on dietary intake, anthropometry, and metabolism in obese Australian adolescents with clinical features of insulin resistance (see chapter 4.2). For this thesis, the author was able to conduct a secondary data analysis to examine the dietary GL and IL. Based on these studies, a set of four analyses has been carried out and their original

articles will be presented subsequently (chapter 5). The four research questions that were addressed are summarized in **Figure 4**.
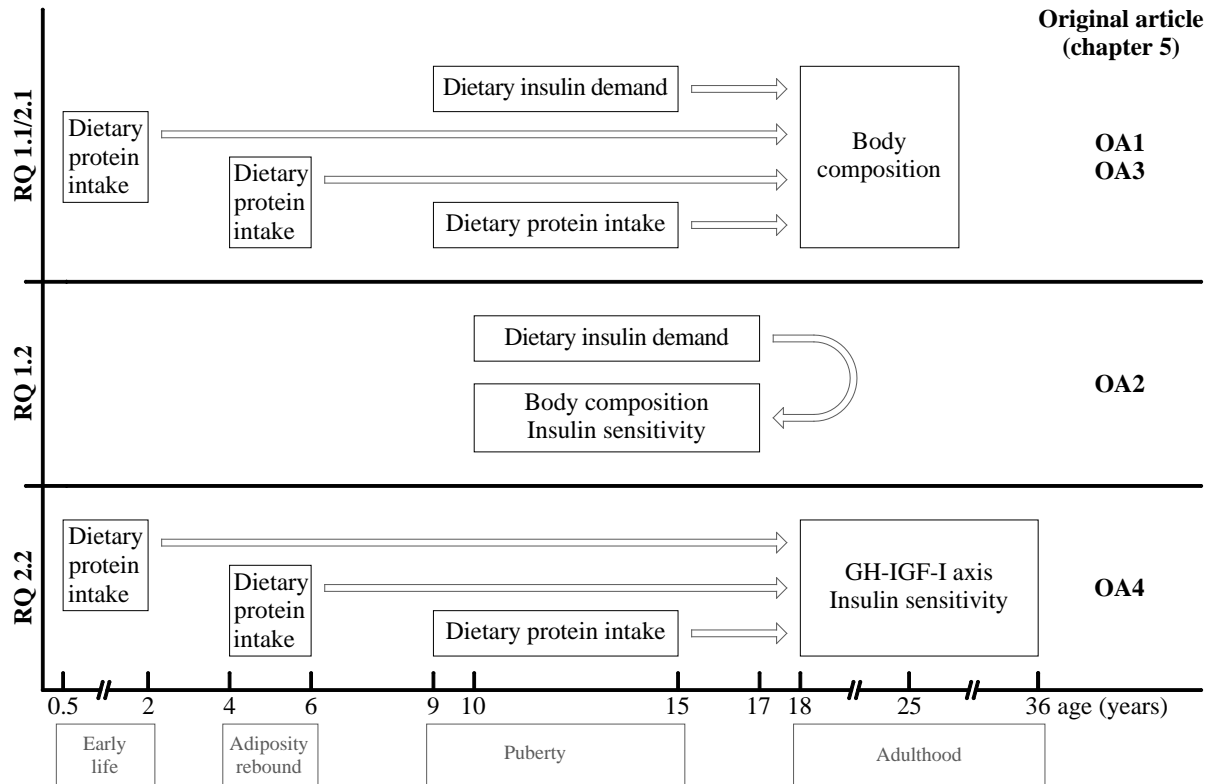
Figure 4 Scheme of research questions over the course of childhood and adolescence. OA, original article; RQ, research question

4. GENERAL METHODOLOGY

4.1   DONALD study

The DONALD study is an ongoing, open cohort study conducted at the Research Institute of Child Nutrition in Dortmund. The major aims of the DONALD study are [317, 318]:

- Analysis of interrelations between dietary intake, metabolism, development and growth
- Determination of intra- and inter-individual trends of dietary intake and nutritional behaviour
- Provision of metabolic reference data from healthy children and adolescents
- Provision of dietary intake data for specific assessments of exposures

Since recruitment started in 1985, detailed data on diet, growth, development, and metabolism have been collected from over 1300 children who are systematically followed up until adulthood. Every year, 35 to 40 healthy infants are newly recruited and first examined at the age of 3 months. Each child returns every 3 months in the first year of life, twice a year in the second year of life and then once annually until adulthood (Figure 5). Assessments include medical examinations, anthropometric measurements, parental questionnaires, and 3-day weighed dietary records. Since 2005, participants are asked to provide fasting blood samples from the age of 18 years, until then the study is purely observational and non-invasive. Moreover, parental information including weight and height is repeatedly assessed [317, 318]. The DONALD study was approved by the Ethics Committee of the University of Bonn, and all examinations are performed with parental and participant's consent.
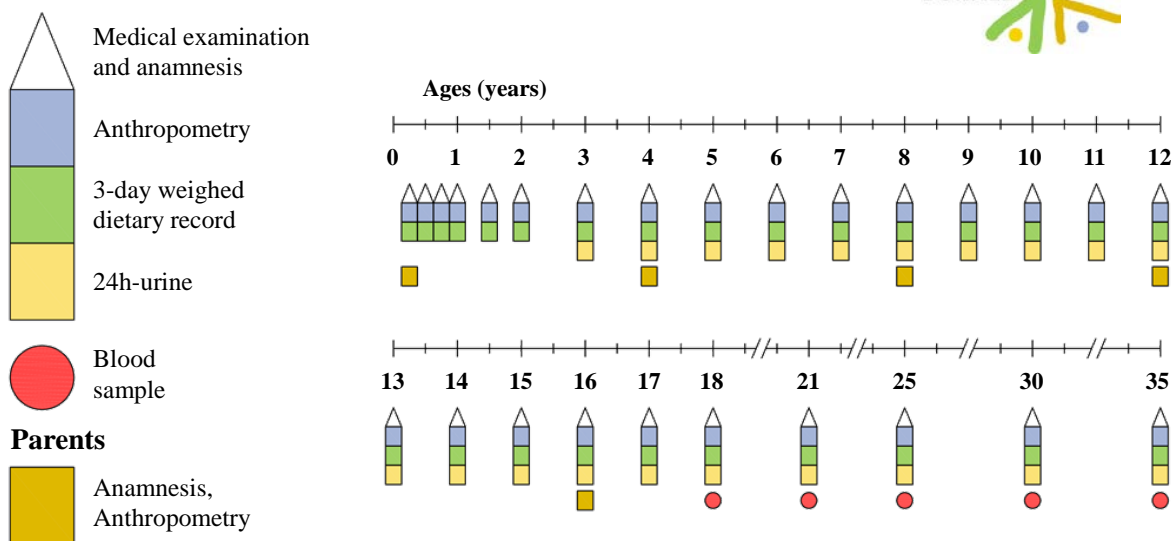
Figure 5 Examination schedule of the DONALD study, from [317]

The 3-day weighed dietary records are analysed using the continuously updated in-house nutrient database LEBTAB (Lebensmitteltabelle) [319], which provides information on energy and nutrients including total protein, animal protein and plant protein. To also allow the examination of dietary GI, GL and insulin demand, the database was extended by the dietary GI according to existing standard procedures [320] and FII, for which a standardized assignment procedure was developed and implemented [321][1]. Composite foods are entered as recipes, therefore, providing comprehensive data on ingredients [319]. In order to create the food groups "dairy products" and "meat products", foods were broken down into their ingredients as appropriate (e.g. pizza was broken down into dairy products, meat products and other product groups). All foods and ingredients were then assigned to their respective food groups, i.e. meat products, dairy products, and miscellaneous.

Venous blood samples are drawn after an overnight fast. IGF-I and IGFBP measurements are not included in the DONALD routine and were measured at the Laboratory of Translational Hormone Analytics in Pediatric Endocrinology Center of Child and Adolescent Medicine at the Justus-Liebig-University in Giessen, Germany. Anthropometric measurements performed in the DONALD study are based on simple measurements only. The estimation of body fat is derived from skinfold measurements. Detailed description of the study methods can be found in the original articles (appendices 1, 3, and 4).

Different, potentially critical, developmental periods comprise the following ages: Early life 0.5-2 years, adiposity rebound 4-6 years, and puberty as 9-14 years for girls and 10-15 years for boys. It should be noted that puberty was defined according to chronological age. Chronological age might be confounded because children of the same age may differ substantially in their pubertal stage. However, the chronological age range we used starts at the same time point at which DONALD participants on average are undergoing puberty according to the age at take-off (onset of pubertal growth spurt). Furthermore, the chronological age range ends where most girls and boys included in the DONALD study have already experienced their first menarche and their voice break, respectively [322, 323]. Therefore, we supposed that chronological age as defined, adequately covers the period of puberty. In addition, preliminary analyses using age at take-off and peak height velocity to define puberty were run and yielded similar results for the relationships of the dietary GI, GL,

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[1] Contribution of Gesa Joslowski: Complementation of assignment of GI values, development and implementation of a standardized procedure to assign FII values to recorded foods, assignment of all FII values to the 3-day weighed dietary records (together with Janina Goletzke)

and insulin demand with %BF. Thus, chronological age was used to define puberty in order to not reduce the sample size too much.

4.2 RESIST study

The RESIST study is a randomised control trial (Australian New Zealand Clinical Trial Registration Number 12608000416392) conducted at the Children's Hospital at Westmead in Sydney. The primary aim of the RESIST study was to determine the effectiveness of two structured lifestyle interventions differing in diet composition on insulin sensitivity in adolescents with clinical features of insulin resistance and/or prediabetes treated with metformin [324].

In total, 111 participants (66 girls) from 10 to 17 years of age were recruited and randomised to either a high carbohydrate, low fat or a moderate carbohydrate, increased protein diet and commenced on metformin. The study is structured in 3 Phases: dietary intervention, intensive exercise, and maintenance phase (**Figure 6**). Assessments include medical examinations, oral glucose tolerance tests (OGTT) and blood tests, anthropometric measurements, questionnaires, and 24h dietary recalls using a standardized three-pass methodology [324]. To assist with estimating the amounts of foods a food model booklet was used [117].The study was approved by The Children's Hospital at Westmead Human Research Ethics Committee (07/CHW/12), Sydney South West Area Health, Western Zone (08/LPOOL/195) and Sydney South West Area Health Service, Royal Prince Alfred Hospital (08/RPAH/455). Written informed consent from parents and assent from the young people was sought prior to their enrolment in the study.

Phase 1 (0-3 months)	At baseline, randomisation to one of two study diets and commencement of metformin treatment, n=111

Allocation to a high carbohydrate, low fat diet (in E%), n=55

Allocation to a moderate carbohydrate, increased protein diet (in E%), n=56

Dietary intervention (medical consultation, dietitian consultation and support, 24hour dietary recall)

Dietary intervention (medical consultation, dietitian consultation and support, 24hour dietary recall)

Phase 2 (4-6 months)

Intensive exercise intervention (Gym sessions: 2 x per week, home based physical activity: 1 x per week, participants continue with their prescribed diet, medical consultation, dietitian consultation and support)

Phase 3 (7-12 months)

Maintenance (Participants continue with their prescribed diet and their exercise routine, medical consultation, dietitian consultation and support)

Figure 6 Design of the RESIST study (modified after [324]). %En, percentage energy

For this thesis, 24h dietary recalls at weeks 6, 9, and 12 were used and analysed using FoodWorks 2009 which uses the Australian Food and Nutrient Database (AusNut) compiled and regularly updated by Food Standards Australia and New Zealand. GI and FII values have been assigned to each food recorded according to standard procedures [321, 325][2]. After an overnight fast, OGTTs were performed and venous blood samples were drawn. Anthropometric measurements are based on single measurements for weight and two measurements for height, using the average value for data analysis. Body composition was analysed using dual-energy X-ray absorptiometry (DEXA). All outcomes were assessed at baseline and after 12 weeks. Detailed description of the study methods can be found in the original article (appendix 2).

All statistical analyses were carried out using the Statistical Analyses System SAS (versions 9.1.3, SAS Institute Inc., Cary, NC). A p-value <0.05 was considered statistically significant. Detailed description of the analytical approaches can be found in the original articles (appendices 1-4).

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[2] Contribution of Gesa Joslowski: Complementation of assignment of GI values, assignment of all FII values to the 24hour recalls (together with Janina Goletzke)

5. ORIGINAL ARTICLES

Aim 1 To examine dietary insulin demand, body composition and weight loss

Nutritional and anthropometric data from the DONALD and RESIST study were used to investigate this aim. Besides GI and GL, the analyses addressed a novel measure of dietary insulin demand. Published GI values were assigned to all carbohydrate containing foods, while published values of the FII were assigned to all foods, i.e. carbohydrate-, protein or fat containing foods, recorded during adolescence in 3-day weighed dietary records in DONALD and 24-hour dietary recalls in RESIST. Percentage body fat (%BF) and BMI in young adulthood were considered as outcome measures for body composition in the DONALD sub-sample. In the RESIST sub-sample, the outcome measures were weight loss expressed as change in BMI %95 centile between baseline and 3months as well as changes in %BF and whole body insulin sensitivity index (ISI) between baseline and 3months.

RQ 1.1 Are dietary insulin demand, glycaemic index, and glycaemic load during puberty prospectively associated with body composition in young adulthood?

The focus of this research question was to examine whether habitual postprandial glycaemic or insulinaemic excursions during the critical period of puberty (girls 9-14 years; boys 10-15years; see chapter 2.2.3) may be of long-term relevance for body composition in young adulthood (18-25 years).

In this study including 262 DONALD participants, dietary GI and GL during puberty were not related to body composition in young adulthood. A higher dietary insulin demand during puberty was associated with higher levels of %BF, but not BMI in young adulthood. The relation to %BF was particularly pronounced in models addressing the effect of substituting carbohydrate- and protein-rich foods with a higher insulin demand for carbohydrate- and protein-rich foods with a lower insulin demand (**OA1** Joslowski 2012).

RQ 1.2 Are the dietary glycaemic load and insulin load associated with weight loss, changes in percentage of body fat and insulin sensitivity in obese adolescents with clinical features of insulin resistance?

This research question addressed the relevance of dietary insulin demand, estimated by GL and IL, for weight loss, %BF and ISI. A secondary data analysis was performed including 91 RESIST participants aged 10-17 years.

Higher dietary GL and IL were associated with less weight loss, i.e. a smaller decrease in BMI %95 centile; however, adjustment for total energy intake attenuated the relation. In addition, conditional models supported a mediation of the association between GL or IL and change in BMI %95 by energy, i.e. that lowering GL or IL facilitated a reduction of overall total energy intake. No relation was observed between dietary GI and weight loss. Dietary GL, GI or insulin demand was not related to change in %BF or ISI. The macronutrient content of the diet (%En) was not related to weight loss, %BF or ISI (**OA2** Joslowski 2013).

For summaries on the respective analysis see the abstracts on the following pages. The original articles can be found in the appendices 1 and 2.

OA1 Prospective associations of dietary insulin index, glycemic index, and glycemic load during puberty with body composition in young adulthood

Joslowski G[3], Goletzke J, Cheng G, Günther ALB, Bao J, Brand-Miller JC, Buyken AE.

Background: Puberty is a so-called critical period for overweight development and characterized by physiological insulin resistance during mid-puberty. This study addressed the hypothesis that habitual consumption of a diet inducing higher levels of postprandial glycaemia or insulinaemia during puberty may have an unfavourable effect on body composition in young adulthood.

Methods: Multivariate regression analysis was performed on 262 DONALD participants with at least two 3-day weighed dietary records during puberty (baseline: girls 9-14years; boys 10-15years) and anthropometric measurements in young adulthood (18-25years). A published dietary glycaemic index was assigned to each carbohydrate containing food. Similarly, each food was assigned a food insulin index (insulinaemic response to a 1MJ portion of food relative to 1MJ of glucose) using 121 values measured at Sydney University.

Results: Dietary glycaemic index or glycaemic load during puberty was not related to body composition in young adulthood. In contrast, a higher dietary insulin index and a higher dietary insulin load during puberty were associated with higher levels of percentage of body fat (%BF) in young adulthood, even after adjustment for early life, socioeconomic and nutritional factors; %BF in energy-adjusted tertiles of dietary insulin index were 22.9 (95%CI: 21.6, 24.1), 24.5 (23.2, 25.7), 24.7 (23.5, 25.9) %, p for trend=0.01; %BF in energy-adjusted tertiles of dietary insulin load were 22.8 (95%CI: 21.5, 24.0), 24.5 (23.2, 25.7), 24.8 (23.6, 26.0) %, p for trend=0.01. Adjustment for baseline %BF attenuated these relationships (p for trend=0.1 and 0.08 respectively). Dietary insulin demand was not related to BMI.

Conclusion: This study suggests a prospective adverse influence of dietary insulin demand during puberty on %BF in young adulthood. Postprandial increases in insulinaemia rather than increases in glycaemia appear to be implicated in an unfavourable development of body composition.

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[3] Contribution of GJ: Complementation of assignment of GI values, development and implementation of a standardized procedure to assign FII values to recorded foods, assignment of all FII values to the 3-day weighed dietary records (together with JG), conduction of statistical analysis, interpretation of the data (together with all co-authors), and drafting of the manuscript

OA2 Dietary glycemic load, insulin load, and weight loss in obese, insulin resistant adolescents: RESIST study

Joslowski G[4], Halim J, Goletzke J, Gow M, Ho M, Louie J C-Y, Buyken AE, Cowell CT, Garnett SP (under revision)

Background & Aims: The optimal dietary approach for weight loss and improving insulin sensitivity in adolescents is unknown. The aim of this study was to explore the association of dietary glycaemic load (GL), insulin load (IL), and weight loss, percentage body fat (%BF), and whole body insulin sensitivity index (ISI) in obese, insulin resistant adolescents after a 3 month lifestyle/metformin intervention.

Methods: Secondary data analysis of 91 adolescents (median age 12.7 years (range 10.1-17.4) participating in an RCT (RESIST; ACTRN12608000416392) who provided at least one 24h dietary recall. Weight change between baseline and 3months was measured by BMI expressed as percentage of the 95th centile (BMI%95). %BF was measured using DEXA. ISI was determined by an oral glucose tolerance test. Linear regression analysis was used to examine the association between diet change in BMI%95, %BF and ISI between baseline and 3 months.

Results: Higher dietary GL and IL were associated with less weight loss (BMI%95), adjusted for sex and pubertal stage (GL: β=0.0466, P=0.007, IL: β=0.0124, P=0.04). Inclusion of total energy intake in the model explained observed associations between dietary GL or IL and change in BMI%95 (GL: P=0.4, IL: P=0.3). Dietary GL and IL were not associated with changes in %BF or ISI. Dietary GI and macronutrient content of the diet (%En) were not associated to changes in BMI%95, %BF or ISI.

Conclusion: Reduced energy diet contributes to weight loss in obese, insulin resistant adolescents. Lower dietary GL and IL diets were associated with a lower energy intake and may hence assist with weight loss.

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[4] Contribution of GJ: Complementation of assignment of GI values, assignment of all FII values to the 24hour recalls (together with JG), conduction of statistical analysis, interpretation of the data (together with all co-authors), and drafting of the manuscript

Aim 2 To examine dietary protein sources, body composition and the GH-IGF axis

Analyses performed in the context of this aim used protein intake data from DONALD participants collected during three potentially critical periods, covering early life, the period of the adiposity rebound and adolescence (see chapter 2.2.3). With regards to the outcomes, the studies examined body composition and the GH-IGF axis in young(er) adulthood.

RQ 2.1 Are different dietary protein sources during childhood and adolescence prospectively related to body composition in young adulthood?

The analysis investigated whether dietary animal or plant protein intake during puberty (girls 9-14 years; boys 10-15 years; n=262) were related to body composition in young adulthood (18-25 years). All foods recorded during puberty were assigned to their respective food groups i.e. meat products, dairy products, and miscellaneous. Furthermore, the association of dietary animal or plant protein intake during early life age (0.5-2 years; n=159) and around the adiposity rebound (4-6 years; n=220) with body composition in young adulthood was investigated. Animal protein did not include protein from human milk.

Among women, a higher dietary animal protein intake during puberty was prospectively associated with higher FFMI, but not with FMI in young adulthood. Among men, a higher dietary animal protein intake was related to higher FFMI and lower FMI, but only after adjusting FFMI for FMI levels in young adulthood and vice versa, i.e. comparable levels of FFMI or FMI, respectively. Examining the sources of animal protein intake, meat, but not dairy protein intakes during puberty were related to higher FFMI in young adulthood among women. No associations were found between dietary plant protein intake during puberty and FFMI or FMI in young adulthood. With regards to early life no relation was found between dietary animal protein intake and FFMI or FMI in young adulthood. A higher dietary animal protein intake around the adiposity rebound tended to be related to higher adult FFMI among men only (**OA3** Assmann 2013).

RQ 2.2 Are different dietary protein sources during childhood and adolescence prospectively associated with the growth hormone-insulin-like-growth-factor axis in younger adulthood?

The last research question addressed whether dietary animal or plant protein intake during early life (0.5-2 years; n=130), around the adiposity rebound (4-6 years; n=179) or puberty (girls 9-14 years; boys 10-15 years; n=213) were related to the GH-IGF axis in younger adulthood (18-36 years). Compared to the research aims considering body composition as an

outcome, this sub-sample was smaller, even though the age range was larger. This is due to the fact that only since 2005, adult participants (aged 18 years or older) were asked to provide fasting blood samples. Analogous to the analysis of RQ 2.1, all foods recorded during puberty were assigned to the respective food groups i.e. meat products, dairy products, and miscellaneous. Animal protein did not include protein from human milk.

Only among women, habitually higher dietary animal protein intake during puberty was related to higher levels of IGF-I, IGFBP-3, and lower IGFBP-2, but not to IGFBP-1 in younger adulthood. In turn, dietary animal protein intake in early life was inversely related to IGF-I levels in younger adulthood among males only. However, no association was observed between dietary animal protein intake around adiposity rebound and IGF-I in younger adulthood. No relations were observed between dietary plant protein intake and GH-IGF axis (**OA4** Joslowski 2013).

For summaries on the respective analysis see the abstracts on the following pages. The original articles can be found in the appendices 3 and 4.

OA3 Prospective association of protein intake during puberty with body composition in young adulthood

Assmann KE, **Joslowski G**[5], Buyken AE, Cheng G, Remer T, Kroke A, Günther ALB.

Objective To examine the association of habitual animal and plant protein intake during the potentially critical period of puberty with body composition in young adulthood.

Methods Multivariable regression analyses were performed on data from participants of the DONALD study with at least two 3-day weighed dietary records during adolescence (girls 9-14 years; boys 10-15 years; n=262), around the adiposity rebound (4-6 years; n=220) or early life (6-24 months[6]; n=159), and anthropometric measurements in young adulthood (18-25 years). Fat-free mass index (FFMI) and fat mass index (FMI) were estimated from four skinfolds.

Results In women, a higher pubertal animal protein consumption was independently related to higher levels of FFMI (p for trend=0.001), but not to FMI (p for trend=0.5). Adjusted means of FFMI in energy-adjusted tertiles of animal protein intake were 15.3 (95% confidence interval: 15.0, 15.5), 15.4 (15.1, 15.7), 16.2 (15.9, 16.6) kg/m². In men, a higher animal protein intake was related to a higher FFMI (p for trend=0.04) and a lower FMI (p for trend=0.001) only after adjusting FFMI for current FMI levels and vice-versa. Plant protein was not associated with body composition among either sex. Higher animal protein intake around the adiposity rebound tended to be related to higher adult FFMI among boys, but not girls. No relations were found between animal protein intake in early life and body composition in young adulthood. Neither plant protein intake in early life or around the adiposity rebound were associated with body composition in young adulthood.

Conclusions Our results indicate that, in women, higher pubertal animal protein consumption yields a higher fat-free mass in young adulthood.

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[5] Contribution of GJ: Compilation of the dataset, support with statistical analysis, and interpretation of the data (together with all co-authors)

[6] Erratum: On page E782, in the second sentence of the last paragraph of the introduction the age in early childhood defined as "12-24 months" is incorrect. It should have read "6-24 months" instead.

OA4 Prospective associations of different protein sources during childhood and adolescence with the growth hormone-insulin-like-growth-factor axis in younger adulthood

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Background Recent studies provide evidence that insulin-like-growth-factor I (IGF-I) and its binding proteins IGFBP-2 and IGFBP-3 are related to the risk of several common cancers. It remains to be clarified whether their levels can be programmed by protein intake from different sources during growth. This study addressed the hypothesis that animal protein intakes during infancy, mid-childhood and adolescence differ in their relevance for the GH-IGF axis in young adulthood.

Methods Data from DONALD participants with at least two plausible 3-day weighed dietary records during adolescence (age girls 9-14 years/boys 10-15 years; n=213), around the adiposity rebound (age 4-6 years; n=179) or early life (age 0.5-2 years; n=130), and one blood sample in young adulthood were included in the study. Parameters of the GH

Results Mean serum levels of IGF-I, IGFBP-1, IGFBP-2, and IGFBP-3 were compared between tertiles of habitual animal protein intake using multivariable regression analysis. Habitually higher animal protein intakes in females during puberty were related to higher levels of IGF-I (p for trend=0.005), IGFBP-3 (p for trend=0.01), and lower IGFBP-2 (p for trend=0.04), but not to IGFBP-1 in young adulthood. In turn, IGF-I levels in young adulthood were inversely related to animal protein intakes in early life among males only (p for trend=0.03), but not to animal protein intake around adiposity rebound (p for trend>0.5).

Conclusion Our data suggest that, among females, a habitually higher animal protein intake during puberty may precipitate an up-regulation of the GH-IGF axis which is still discernible in young adulthood. By contrast, among males, higher animal protein intakes in early life may exert a long-term programming of the GH-IGF-I axis.

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[7] Contribution of GJ: Conduction of statistical analysis, interpretation of the data (together with all co-authors), and drafting of the manuscript

6. GENERAL DISCUSSION

The overall aim of this thesis was to investigate the relevance of dietary insulin demand and dietary protein intake during adolescence for the development of body composition and/or the adult GH-IGF axis. In the following chapters, the central findings of the conducted studies, based on the aims and research question presented in chapter 3, will be discussed. Consecutively, the general methodological issues concerning the DONALD and RESIST study will be discussed. Finally, public health considerations will be presented.

6.1   Research aims

In this chapter, the findings are discussed with respect to the research questions (see chapter 3) and their common scientific background. Since this thesis is cumulative, more detailed and specific discussion can be found in the original articles (for details see appendices 1-4).

6.1.1 Aim 1 To examine the dietary insulin demand, body composition and weight loss

The **first research question** (RQ 1.1) of aim 1 addressed whether the dietary insulin demand, glycaemic index, and glycaemic load during puberty were prospectively associated with body composition in young adulthood and was addressed by OA1 (appendix 1).

Insulin demand has so far never been related to body composition and with this analysis a new link between dietary insulin demand and body composition has been demonstrated. The finding that a higher dietary insulin demand, but not dietary GI or GL, was related to higher levels of body fat in young adulthood indicates that postprandial rises in insulinaemia rather than glycaemia may have adverse consequences for the development of adult body composition. This is in line with animal and human studies [38, 326], suggesting that early postprandial insulin response (at 30 min) might be related to a higher obesity risk. Potential mechanisms including redirection of nutrients away from oxidation in the muscle and towards storage in fat [176], suppression of lipolysis [161], and reduction of insulin sensitivity [13] seem plausible (see chapter 2.2.1). Still there exists controversy about insulin response being causal for obesity and a heated debate has also entered the day press [327]. Under the hypothesis that insulin response is causal for obesity, Gary Taubes claims that the "lipophilia hypothesis" explains overnutrition; his essay was recently published in the British Medical Journal [42]. Foods stimulating insulin levels, especially carbohydrate rich foods, trigger the development of obesity, because they cause a hormonal response that drives fat accumulation. Hence, overeating is a compensatory response to lipophilia rather than a cause. Furthermore,

Taubes rejects the energy balance hypothesis, which states that overnutrition is causal for obesity [42]. The essay has been controversially debated [328-332]. Yet, it is not clear whether hyperinsulinaemia is a cause or an effect of obesity, but our data provide some support for an involvement of hyperinsulinaemia within obesity development.

There exists evidence that hyperinsulinaemia, at least in early stages, may be a physiological adaptation to obesity helping to limit further weight gain [41, 333, 334]. On the other hand, suppression of insulin response using octreotide (a somatostatin analog) among 44 severely obese adults with a mean age of 39 years was associated with loss in weight and fat mass as well as concomitant decrease in total energy intake [335], suggesting that hyperinsulinaemia may be a risk factor for developing obesity. In addition, among 5-9 year old children from a Pima Indian population (n=328) those with insulin resistance gained more weight than insulin-sensitive children after a 10 year follow-up [336]. However, this is only indirect support as this study did not provide data on insulin secretion. The debate whether insulin response is related to obesity has not been resolved yet [43]. Nevertheless, our observed associations may provide an explanation why observational and clinical trials among children and adolescents did not find a strong relation between GI or GL and body composition and weight loss (see chapter 2.3.1), since dietary GL may perhaps capture the dietary insulin demand less precisely compared to the dietary II/IL. Nonetheless, results need to be confirmed in other populations.

Children and adolescents with an increased BMI may be more vulnerable to a higher insulin demand. Results from the DONALD study showed that overweight adolescents with a higher dietary GI at baseline tended to have higher %BF and BMI SDS at baseline, while no association was observed for normal weight adolescents [279]. Thus, a relevance of insulin demand among obese and insulin resistant adolescents is conceivable. In this regard, findings from Papadaki et al are interesting as they show that a diet high in dietary protein and low in dietary GI was related to decreased rates in overweight or obesity among children and adolescents at risk for overweight [25] (see also chapter 2.3.1). A high protein and low-GI diet is characterised by a lower insulin demand because of partial substitution of insulin demanding carbohydrates for less insulin demanding proteins (particularly non-dairy protein). Hence, it is of interest to examine the relation between dietary insulin demand and body composition among an obese population.

The **second research question** (RQ 1.2) of aim 1 addressed the association of the dietary glycaemic load and insulin load with weight loss, changes in percentage of body fat and

insulin sensitivity in obese adolescents with clinical features of insulin resistance. In contrast to what has been observed among participant of the DONALD study, we did not find an independent association between dietary insulin demand and weight loss or body composition as has been discussed (for details see OA2, appendix 2). Similarly, dietary insulin demand among the RESIST participants was not independently related to weight loss after 6 months (n=83, data not shown). The observation that the association between dietary GL or IL and weight loss might be mediated by energy, i.e. that lowering GL or IL facilitated a reduction of overall total energy intake, is in line with the results of the RESIST study's intention-to-treat-analysis, as briefly described in the following paragraph.

Ninety-eight participants (58 girls) completed the 6-month intervention. Both dietary interventions and metformin treatment resulted in a decrease in BMI %95 centile after 3 months (p for difference<0.001). BMI %95 centile also decreased significantly between 3 and 6 months (p for difference=0.009) and remained different from baseline. However, no differences were seen between diet groups at any time point. Results for %BF have not been reported thus far. After 3 months of dietary interventions and metformin treatment, the mean ISI had increased by 0.3 (95%CI 0.2, 0.4; p for difference<0.001), but no differences were seen between diet groups. Garnett et al discuss that a lack of observed differences among the outcomes could stem from the poor dietary adherence or limitations of monitoring and reporting in both diet groups. In addition, this trial was undertaken in a real-life setting and the difficulty the adolescents had in altering the macronutrient content of their prescribed diet may be a consequence of readily available high-carbohydrate snack foods [337].

Using data from RESIST, we were able to also examine insulin sensitivity as an outcome, but did not see any relation between insulin demand and change in ISI. As mentioned in the original article (OA2), some studies report an effect between lower dietary GL and GI on decreased insulin resistance during a 6 months intervention period [284, 338], while others did not find an effect of a lower dietary GL and insulin resistance after a 6 months [339] and 2 year intervention [285]. As described in the previous paragraph, insulin sensitivity improved during 3 months of intervention. However, a 3 months intervention might not have been sufficient to detect a relation between dietary GL or IL and ISI. Among 226 participants of the DONALD study, a higher dietary GI during puberty was prospectively related to higher insulin resistance in younger adulthood [340]. Moreover, higher pubertal protein intake, but not insulin demand, tended to be related to higher insulin resistance in younger adulthood as will be discussed below (chapter 6.1.2). Hence, it is conceivable that there exists a long-term

relevance of dietary factors, including GI and protein, on insulin sensitivity resulting from greater demands on β-cell function.

Overall, insulin demand has never been related to weight loss. Although the findings with regard to dietary insulin demand and body composition from the DONALD study (OA1) seem plausible, the results should not be overinterpreted. This is underpinned by the fact that no independent relation between insulin demand and %BF or weight loss was found in the RESIST study. To date, data does not support any dietary recommendations for insulin demand, but as suggested by the qualitative approach in the DONALD analyses, a specific relevance of insulin demand with respect to body composition is conceivable. Even though the analyses of the RESIST study suggest that lowering energy intake is more relevant for weight loss, it can be speculated that reducing the insulin demand of the diet might be an alternative to help reducing energy intake, since the conditional models suggested that total energy intake may mediate the relation between dietary GL or IL and change in BMI %95 centile. There exists the possibility to exchange foods with a high FII for foods with a low FII or to have one serving of a food with a low FII within a meal, similar to what is done for GI.

Table 8 provides some examples of potential food exchanges mainly among carbohydrate rich foods. Among these, a lower FII may result from lower GL, but also higher protein and/or fat contents of a food. The exchange of carbohydrate rich foods for protein rich foods would in general result in a rather lower dietary insulin demand, because protein rich foods tend to have a lower FII (e.g. poached eggs: FII=23; tuna canned in water: FII=26; beef steak: FII=37; but also lentils served in tomato sauce: FII=42; canned Navy beans: FII=23 [17]). Even though protein rich foods result in a postprandial insulin response, especially the co-ingestion of protein and carbohydrates [16, 72], but also fat [14], may result in increases of postprandial insulin response. Of course, an exchange of foods as described would only be feasible if the whole diet is considered [341], i.e. not only focussing on FII. For example, this includes a limited intake of foods containing saturated fat, added salt, added sugars and alcohol.

Table 8 Potential food insulin index exchange list[1]

Instead of...	FII	Choose...	FII	Explanatory
BREAKFAST				
Cornflakes (Kellogg Foods Inc. USA, Wells Fargo Bank, MN)	82	**Porridge** (Uncle Toby's Inc. Nestle Pty Ltd, Sydney, Australia)	29	Products with a lower GL and higher protein and fat content have a lower FII
		or **100% Natural Granola Oats, Honey Raisins** (Quaker Oats Inc. Chicago)	41	
Skim-fat milk (Dairy Farmers)	60	**1% fat milk** (Dairy Farmers)	34	Products with a higher fat content have a lower FII
LUNCH				
Potatoes (russet boiled, peeled; Australia)	88	**Lentils, served with tomato sauce** (Australia)	42	Products with a lower GL and/or a higher protein and fat content have a lower FII
		or **Brown pasta** (both Sam Remo, Australia)	29	
SNACKS				
Bananas (raw, peeled, Australia)	59	**Apple**, Red Delicious, raw (Australia)	43	Products with a lower GL have a lower FII
Mars bar (Mars Confectionary Inc. Auburn, Australia)	89	**Hershey's milk, chocolate** (Hershey Foods Inc. Hershey, PA)	34	Products with a lower GL and higher fat content have a lower FII
		or **Snickers bar** (Masterfoods, Hackettstown, NJ)	37	
DINNER				
White bread (Sunblest; Tiptop Pty Ltd, Enfield, Australia)	73	**Grain bread** (Tiptop Bakeries Inc. Australia **or** Burgen, Soy-Lin, Chatswood, Australia)	41	Products with a lower GL and higher protein and/or fat content have a lower FII
Whole meal bread (Riga Bakeries Inc. Sydney, Australia)	70		52	

[1] All FII values were measured among 10 healthy individuals [17]. FII, Food insulin index

6.1.2 Aim 2 To examine dietary protein sources, body composition and the GH-IGF axis

Whether different dietary protein sources during childhood and adolescence were prospectively related to body composition in young adulthood was considered by the **first research question** of aim 2 (RQ 2.1). Earlier findings of the DONALD cohort suggest that a higher habitually animal protein intake, but not plant protein intake, in early life (12 months of age) and around the adiposity rebound (5-6 years) was associated with an unfavourable body composition at the age of 7 years, i.e. a higher BMI SDS and %BF (n=203) [342]. This analysis did not provide information about fat mass and lean body mass in specific in order to disentangle a potential differential relation of animal protein intake to fat mass and lean body mass.

The question remains whether the association between dietary animal protein intake in early life and body composition in childhood is transitory or whether it extends into adulthood. The

analysis enclosed in this thesis (see OA3, appendix 3) addressed the long-term relevance of an early animal protein intake (0.5-2 years) for body composition in young adulthood (n=157) and did not find a relation; thus suggesting that a relation may not extend into young adulthood. By contrast, higher animal protein intake around the adiposity rebound (4-6 years) tended to be related to higher adult FFMI among boys (n=107), but not girls (n=113), indicating a relation towards higher lean body mass in young adulthood. Nevertheless, the analyses are limited by small sample sizes and need to be interpreted with caution. Similar to what has been observed by Günther et al [342], no relations were found between plant protein intake in childhood and adolescence and body composition in young adulthood. This might be explained by the fact that plant protein contains less essential amino acids, which are important for anabolic processes of lean body mass and lower bioavailability compared to animal protein [170, 343] – a circumstance, which also applies to the associations examined in puberty (see below).

In addition, higher animal protein intake during puberty was related to higher FFMI in young adulthood (for details see OA3). Earlier results from the DONALD study showed that a higher dietary insulin demand during puberty was associated with higher FMI in young adulthood, even though adjustment for baseline FMI attenuated the relation (similar to the observation regarding %BF, see OA1). These findings demonstrate a relevance of puberty for the development of body composition, although relations may be different with regards to exposures. Dietary animal protein intake during puberty seemed to be favourable for an increase in lean body mass, whereas the dietary insulin demand during puberty was related to higher levels of fat mass. These different observations might be partly explained by the fact that the insulin demand of a diet is indeed driven by protein, but primarily by carbohydrate intake. The relevance of differences between sources of dietary animal protein, i.e. dairy and meat protein will be discussed below in more detail.

Controversy exists on the relation of protein intake with body composition or weight loss between results from observational studies and clinical trials among adults (see also chapter 2.3.2). Body weight and BMI are only a proxy measure for %BF. In fact, BMI can reflect both a higher lean body mass and a higher %BF, which is important to keep in mind when examining relations between dietary protein intake and BMI. This has been elegantly demonstrated by a recent randomised controlled trial among 25 healthy young adults aged 18-35 years examining the effect of overeating among 3 different protein groups: 5%En from protein (low protein), 15%En (normal protein) or 25%En (high protein). Data was collected under rigorous experimental conditions in a metabolic ward. Weight gain and the increase in

BMI in the low protein group was about half of that in the normal and high protein group. On the other hand, this trial showed that the increase in %BF was similar among all diet groups and that the gain in weight and BMI was particularly resulting from an increase in fat-free mass only [296]. Therefore, considering solely the gain in weight or BMI would incorrectly suggest an unfavourable effect of protein on body composition.

In the observational analysis enclosed in this thesis we were able to distinguish between fat mass and fat-free mass showing that higher animal protein intake may not necessarily be adverse with regard to body composition, but in fact increase lean body mass. Overall, our results are broadly in line with findings from observational studies and randomised controlled trials among children and adolescents (see chapter 2.3.2 and OA3). With regards to FFMI, prospective relations between higher protein intake at the age of 8-10 years and higher FFMI after 6 years of follow-up were observed among girls in the 5th BMI quintile [298]. Taken together, it is important to consider both fat mass and fat-free mass when examining relations between dietary protein intake and body composition and there may exist a specific relevance among girls. For boys, hormonal influences during puberty may be more important than differences in protein intakes as discussed in the original article. With respect to FMI, statistical power might have been insufficient, as mentioned in the original article. Therefore, it cannot be ruled out that there may exist a relation between dietary animal protein intake with FMI, possibly inverse, as was found in other prospective studies [297, 298].

Concerning the sources of pubertal animal protein intake among females, the variance for meat protein intake was greater than for dairy protein intake (σ^2=2.2 vs. σ^2=1.4; data not presented in OA3) and the actual differences between the highest and lowest tertile were 1.7%En vs. 4.6%En for meat and 3.0%En vs. 5.1%En for dairy protein intake (data not presented in OA3). This may explain why we did not observe a relation with regards to dairy protein intake. In addition, milk has been shown to result in high insulin responses [58, 67] and a high dairy protein intake may therefore reflect a higher insulin demand compared to meat protein intake. While higher meat protein intake is related to higher FFMI, it is thus conceivable that dairy protein intake might be related to FMI, but not FFMI. However, statistical power might not have been sufficient to detect a relation with regards to FMI as mentioned above.

No relation between dietary plant protein intake during puberty and body composition in young adulthood was observed. The overall proportion of plant protein intake was relatively low, which might explain why no associations were observed with body composition (median

plant and animal protein intakes among females were 4.8%En and 7.9%En, respectively and 4.8%En and 8.4%En, respectively, among males). As already mentioned, plant protein contains less essential amino acids than animal protein, for details on potential mechanisms see the original article (OA3), which might further explain the lack of an association.

These results suggest that dietary protein intake in childhood, and in specific the early life, may not be as relevant for adult body composition as puberty. Besides the greater time lag between early life or childhood and young adulthood, puberty is in general a period of change in which adolescents start being more independent from their parents and are influenced by their social environment including their peer group, school, and life style factors. Moreover, this goes hand in hand with changes in health behaviour which may persist into adulthood [344, 345]. Hence, it is possible that a nutritional pattern developed during adolescence is more relevant for the later life compared to childhood.

The **second research question** (RQ 2.2) of aim2 was to gain insight whether different dietary protein sources during childhood and adolescence were prospectively associated with the GH-IGF axis in younger adulthood. Besides its role in childhood growth and metabolism, it has been hypothesised that there may exist an early programming of the GH-IGF axis, i.e. an inverse association between (animal) protein intake in early life and IGF-I levels in young adulthood (a resetting of the GH-IGF axis; see chapter 2.3.3). In other words, a high early protein intake may down-regulate the GH-IGF axis in the long-term. Our results partly support this hypothesis and are mostly in accordance with earlier studies from Ben-Shlomo and Martin et al [31, 32]: We found that animal protein intakes in early life were inversely related to IGF-I levels in younger adulthood. This result was, however, confined to males only. No relations were observed between early plant protein intake and GH-IGF axis in women or men in younger adulthood (see OA4, appendix 4).

Moreover, an analysis of a Dutch sub-sample of the European Prospective Investigation into Cancer and Nutrition study provides evidence on the long-term effect of nutrition on the GH-IGF axis. Among a small sample of 87 postmenopausal women, caloric restriction (famine) at the ages of 2-20 years was prospectively related to higher IGF-I and IGFBP-3 concentrations in later life (age 52-69 years) [346]. This result suggests an up-regulation of the GH-IGF axis in adulthood in response to caloric restriction and is compatible with our finding which indicated a down-regulation of the adult GH-IGF axis in response to high animal protein intakes accompanied with adequate energy intake in early life. It could be argued that energy intake may play a primary role for this early programming, but additional adjustment for

energy intake in early life did not alter the relation between high animal protein intake in early life and lower IGF-I younger adulthood (data not shown in OA4). To date, it is not clear which mechanism lies behind this programming.

This resetting hypothesis was not confirmed for breast feeding using data of the DONALD study; in neither women nor men, breastfeeding duration was associated with mean adult concentrations of IGF-I, IGFBP-1 or IGFBP-3 [347]. Animal protein intake in our analysis did not include breast milk. When comparing breast milk to infant formulas and cow's milk, protein content in breast milk is lower. In fact, breast milk contains on average 1.13g (min, max: 1.03, 1.43) protein per 100g [81], while the European guideline for infant formula based on cow's milk protein claims a protein content of 1.26-2.1g per 100g infant formula and 1.26-2.45g per 100g follow-on formula[8] [348]. This guideline is relatively new, however, older German and European guidelines claim a comparable or slightly higher protein content for infant formula based on cow's milk protein, respectively (i.e. the German reference: 1.2-1.9g per 100g until 31. May 1994 and the European guideline: 1.58-2.1g per 100g since 1. December 1992[9]) [349], which is of note, as all participants were born between 1971 and 1993. In this comparison cow's milk has the highest protein content, i.e. 3.32g protein (min, max: 3.08, 3.70) per 100g [81]. With respect to essential amino acids, lower concentrations are found in human breast milk compared to cow's milk. In fact, concentrations of leucine, isoleucine, and valine are around 2.7 times higher in cow's milk compared to breast milk. Similarly, the content of arginine and lysine is 2.3 and 3 times higher in cow's milk compared to human breast milk [81]. Therefore, it can be speculated that the protein content of breast milk may be too low to play a role within programming of the GH-IGF axis.

While no associations of animal protein intake around the adiposity rebound with IGF-I in younger adulthood were seen, a habitually higher animal protein intake during puberty was related to higher levels of IGF-I, IGFBP-3, and lower IGFBP-2, but not to IGFBP-1 in younger adulthood among women only. Due to the time lag between the adiposity rebound and younger adulthood, one would rather expect to see a relation between pubertal diet and adult GH-IGF axis. Furthermore, puberty may also be a critical period for the development of the GH-IGF axis, similar to what has been observed for body composition. Especially girls, who have a higher degree of physiological insulin resistance during puberty [3], may be more vulnerable to dietary effects on the GH-IGF axis than boys, as has been discussed in the

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[8] Data per 100 kcal were converted under the assumption that infant formula contains 70 kcal/100g to make comparisons with other guidelines possible

[9] Data for energy density of 70kcal/100g

original article. Initially, we also examined associations of the pubertal dietary insulin demand with the GH-IGF axis. A trend was observed that pubertal insulin demand was related to IGF-I, but it emerged that this association was driven by dietary protein intake. Thus, trends between dietary insulin demand and IGF-I might be explained by the fact that protein contributes to the dietary insulin demand.

Potential mechanisms relating dietary animal protein intake and higher IGF-I may work through amino acids (for details see OA4). This is supported by the finding that higher pubertal meat, but not dairy, protein intake was prospectively related to higher IGF-I concentrations in younger adulthood, again, only among women. Even though dairy and meat products both contain amino acids, their contents differ between them. Of note, IGF-I is not only stimulated by essential amino acids [208], but also by non-essential amino acids such as glutamine [209], arginine [210] or the combination of amino acids (lysine and arginine) [211].

On the other hand, no prospective associations were observed for pubertal intakes of plant protein and the GH-IGF axis in younger adulthood. Similar to what has been observed among the DONALD sub-sample used to examine protein intake and body composition, the overall proportion of dietary plant protein intake was relatively low (median plant and animal protein intakes among females were 4.8%En and 7.9%En, respectively and 4.8%En and 8.4%En, respectively, among males) and thus explanations given above might also apply in this regard.

Results of our analysis showed, that among women, higher pubertal animal protein intakes were related to lower IGFBP-2 concentrations in younger adulthood, hence reflecting lower insulin sensitivity [212]. In line with this, a tendency between higher pubertal animal protein intake and higher levels of insulin resistance (HOMA-IR) among adult women was observed (data were not shown in OA4). By contrast, no relations were found between dietary plant protein intakes during puberty and IGFBP-2 or HOMA-IR in younger adulthood. These findings are in line with results from prospective cohort studies among adults [350, 351] and potential mechanism have been already discussed (OA4). As previously described, higher protein intakes may be beneficial for weight loss and body composition at least over the short-term [19, 352]. Hence, it is possible that high protein intake and induced weight loss may compensate negative effects of high protein intake on insulin sensitivity in the short-term [352]; however, long-term effects are less clear.

6.2 Methodological considerations

Analyses in this thesis were based on sub-samples of the DONALD and the RESIST study. In the following chapters, general methodological issues concerning these studies will be discussed. The focus will lie on the study populations, the dietary assessment and estimating dietary intake, anthropometric and blood measurement which built the basis for the exposure and outcomes variables investigated.

6.2.1 Study populations

The DONALD and RESIST study comprise both a longitudinally designed cohort study and an intervention study. Even though a longitudinal, prospective cohort study is purely observational, a group of individuals can be followed over time and it is possible to study different exposures in order to determine how these factors are related to specific outcomes. By contrast, an intervention study examines the direct effect of a random or non-random assigned exposure on an outcome. However, the analysis included in this thesis was secondary data analysis of the RESIST study and hence also purely observational.

DONALD Study

The prospective and longitudinal nature of the DONALD study entails the possibility to investigate periods from 3 months of age until adulthood. This design is superior to cross-sectional studies which can only assess an association at a certain time point. Prospective cohort studies are able to identify occurrences of diseases and/or their development. Furthermore, risk factors for specific populations can be identified and give clues of possible causal relations [353].

The DONALD study is an appropriate but non-representative sample, as discussed in the original articles (OA1, OA2, and OA4). Only Caucasians are included and participants display a higher education and a generally high interest in nutrition and health-related topics [318]. However with regards to anthropometrics, former comparisons of the BMI distribution in the DONALD study with the German reference population did not suggest major deviations [354]. DONALD participants included in the analyses of this thesis had slightly lower or comparable BMI values during puberty compared to the German reference population [92]. Furthermore, DONALD participants appeared to have slightly higher BMI values during early life and comparable BMI values around the adiposity rebound than the German reference population [92].

Participants included in the DONALD sub-sample for the body composition analyses (OA1 and OA3) had a median %BF of 17% for men and 29% for women in young adulthood (median age was 19 years (the age range was 18 to 25 years)). Among men, 30.3% were overweight and 4.1% obese; among women, 12.2% were overweight and 4.3% obese. Similarly, men and women who were included in the DONALD sub-sample for the IGF analysis (OA4) had a median %BF of 18% and 31% in younger adulthood (median age was 22 years (the age range was 18 to 36)), respectively. Among men, 33.7% were overweight and 7.4% obese; among women 17.8% were overweight and 5.9% obese. These prevalence are lower, especially for women, than compared to the results of the German Health Interview and Examination Survey for Adults (DEGS1; conducted from 2008 to 2011) for 18-29 year old men and women where 35.3% of men and 30.0% of women were overweight, 8.6% of men and 9.6% of women were obese [113]. This large nationwide survey used BMI to identify overweight and obesity since it can be measured relatively quickly, easy and highly standardised compared to other indicators of overweight. Therefore, no data on %BF were available.

The KiGGS study included a nutrition module, so-called EsKiMo (Ernährungsstudie als KiGGS Modul) [355], providing representative data on dietary intake among children and adolescents. The median daily macronutrient intakes during puberty were 13%En protein, 51%En carbohydrates, and 36%En fat among girls and boys in the DONALD sub-samples (median age was 12 years). Therefore, dietary protein intake of boys and girls was comparable to that of 12 year old boys and girls in EsKiMo. The dietary carbohydrate intake in the DONALD sub-samples was a bit lower for boys and girls (i.e. 1%En for boys and 1.5%En for girls), while dietary fat intakes were higher for boys and girls (i.e. around 3%En for boys and girls), compared to the results of EsKiMo. The median energy intake in the DONALD samples was around 9MJ for boys and 7MJ for girls, thus around 1MJ lower compared 12 year old boys and girls in EsKiMo.

Longitudinal cohort studies may in general have the problem of non-representativeness, as only participants who are really interested in a study will participate over the long-term and this may be often associated with a higher education and socioeconomic status. The DONALD study focusses on details, as it includes repeated, closely spaced measurements, which allow the investigation of relations between habitual dietary intakes in childhood and adolescence (see also chapter 6.2.2, *Dietary assessment*) and health-related outcomes later in life. With these multiple assessments during growth, potentially critical developmental periods for later disease risk are covered and made it possible to consider them within the

analyses, i.e. early life, adiposity rebound and puberty. This is an advantage compared to other large studies, which are less detailed.

RESIST study

The RESIST study included obese adolescents with clinical features of insulin resistance and/or prediabetes. This is a clinical population at risk of developing type 2 diabetes and other chronic diseases, not meant to be representative for the general population.

The vast majority of the RESIST participants (91%) were born in Australia, but only 27% of participants (n=30) reported having both parents born in Australia [337]. Of these, 6 had at least 1 parent who was an Aboriginal or Torres Strait Islander. The country of birth of the remaining participants' parents included North African/Middle Eastern (16%), Southern/Central Asia (12%), Southern/Eastern Europe (8%), New Zealand (Maori)/Pacific Islands (6%), and South American (6%). One fifth (20%) reported speaking a language other than English at home. In addition, most participants (87%) reported a family history of obesity [337].

The secondary data analysis included in this thesis was not clearly prospective. This is due to the fact that the average dietary intake was calculated from 24h dietary recalls at weeks 6, 9, and 12 to examine associations with outcomes at 3 months and not all participants completed 3 recalls. It is not possible to draw a conclusion with regards to cause and effect, as discussed in the original article (see OA2). On the other hand, the RESIST sample is the sample of particular interest. RESIST participants are obese and have features of clinical insulin resistance and/or prediabetes, thus they are at risk of developing type 2 diabetes. Especially among adolescents, development of type 2 diabetes is of concern as complications are common and may appear early in children and adolescents with type 2 diabetes [356, 357]. The recruitment of this sample was difficult and it is therefore remarkable that overall 96% and 88% of participants completed the 3 and 6 months visit of the study, respectively [337].

With both studies and their specific characteristics it was possible to study and answer the underlying research questions of this thesis (see chapter 3). Even though the studies are non-representative, it should be noted that representativeness is of minor importance when examining internal associations between exposure and outcome, since it does not affect internal validity. Unquestionably, the results cannot be generalised. In addition, the study samples drawn from the DONALD and RESIST study were both relatively small. This specifically applies to the sub-samples of the DONALD study examining early life and the adiposity rebound (OA3 and OA4) and hence statistical power might not have been sufficient.

With regards to the secondary data analysis of the RESIST study, explanatory power may be limited due to the small sample size and the not clearly prospective design.

6.2.2 Data assessment

Dietary assessment

The doubly labelled water technique is considered the standard reference and therefore the "gold standard" for measurement of total energy expenditure in humans, but it is of seldom use due to high cost and high facility requirements [358]. In the DONALD study the dietary intake is assessed using 3-day weighed dietary records, which is often regarded to be the "gold standard" within traditional dietary assessment methods [359].

Weighed food records do not rely on an individual's memory and portion sizes are very precise as they do not rely on estimations. Information of type and brand name of all foods consumed is requested. In the DONALD study, recipes are collected as well as the packages or the food labels for commercial foods consumed. These additional information are then added to the dietary record data [318]. Disadvantageous of weighed diet records and prospective methodologies in general are the high burden to participants and participants need to be motivated. Due to the act of weighing and recording food intake in prospective methods, participant's food choices may be influenced during the recording period and hence the method is reactive [360]. In addition, a 3-day dietary record may not be able to capture foods which are seldomly eaten such as nuts or fish and repeated 3-day weighed dietary records are needed to capture the habitual dietary intake [361].

Furthermore, DONALD participants grow up with this method and detailed data on dietary intake are assessed repeatedly. As discussed earlier, interest in the study may be due to the higher socioeconomic and educational status of the DONALD population and an excellent example of the motivation and compliance among DONALD participant's is the collection of dietary records during puberty. Puberty is a phase of change and development for adolescents, which may not be the easiest time to impose high standards in terms of data collection. It is therefore of note that DONALD participants included in the analyses provided 88% (OA1 and OA3) and 86% (OA4) of the maximum number of food records which had been scheduled. Additionally, 94% (OA1 and OA3) and 93% (OA4) of all 3-day weighed dietary records completed were plausible. A 3-day weighed dietary record was considered plausible when the total recorded energy intake was adequate in relation to the basal metabolic rate (estimated from the Schofield equations [362]) using modified cut-offs from Goldberg et al [363]. A

validation study among DONALD participants has shown that dietary protein intake in children and adolescents can be estimated with acceptable validity by 3-day weighed dietary records [364], suggesting good validity of dietary data.

The RESIST study used 24h dietary recalls to estimate participant's intake of food and beverages. This method has the ability to collect detailed, qualitative information about foods consumed with lower burden for participants. Therefore, it is applicable to broad populations of different ethnicity, can be conducted successfully either face-to-face or over the phone. Disadvantageous of 24h recalls are that they rely on memory, perception, conceptualization of food portion sizes and the presence of an observer [365]; in the RESIST study, 24h dietary recalls were conducted by trained dieticians. To capture the habitual intake of a population repeated 24h dietary recalls are needed. For the RESIST study this was the appropriate method to assess dietary intake and suitable for this young study population. It has been previously used in a nationwide study among Australian adolescents and a food model booklet was applied to assist with estimating the amounts of foods [117]. The analyses of the DONALD study included only participants who had provided plausible or more plausible than implausible food record data. With regards to the RESIST study a plausibility check based on basal metabolic rate was not an option because this study was designed as a weight loss study. Therefore, all 24h dietary recalls provided were included in the analyses. Overall, RESIST participants included in the secondary data analyses provided 78% of all scheduled recalls (i.e. 13 participants provided 1 recall, 34 participants provided 2 recalls, and 44 participants provided 3 recalls), thus less diet data than that available from the DONALD participants.

Estimating dietary intake

Concern has been raised regarding the reproducibility of measuring the GI value of a food [36], because there exist numerous factors influencing the glycaemic response to a food (see chapter 2.1.1, **Table 1**). Furthermore, the GI can vary between similar foods due to regional or seasonal differences. Variability of glycaemic response is however, not only a problem of GI, but of other nutrients as well [51, 366]. As already discussed in the original articles (OA1, OA2), estimation of dietary GI and GL as well as II an IL is challenging. The GI assignment is often difficult using FFQs, due to the problem that low and high-GI foods end up in one food group (e.g. whole-kernel and whole meal breads). In addition, assignment of GI values to foods is often based on GI values available for similar foods only and may vary from researcher to researcher. Considering these problems, some studies may not be able to validly

discriminate consumers of diets with a high dietary GI from those consuming a lower GI diet [367]. By contrast, 3-day weighed dietary records as well as 24h dietary recalls provide detailed data on reported foods allowing the assignment of GI and FII value to each (carbohydrate containing) food recorded. Nevertheless, assignments of GI values to foods recorded may vary and it is hence important to follow standard procedures (see OA1 and OA2). Finally, the method of estimating GI and GL of a whole diet has been criticised [36], since the glucose response is known to be influenced by proportions of macronutrients in a mixed meal. However, many, but not all studies [368, 369], suggest that the estimation of the GI of a whole diet or mixed meal can be accurately estimated from GI values of the constituent foods [41, 370-372]. Consequently, limitations raised for GI may also apply to the estimation of dietary insulin demand (as mentioned in OA1).

Anthropometric measurements

In both studies, DONALD and RESIST, anthropometric measurements were performed according to standardised procedures by trained personnel. Skinfold measurements were used within the DONALD study to estimate %BF, whereas the RESIST study used the DEXA method to estimate %BF, fat mass and lean mass.

Even the best methods used in epidemiology and clinical trials are indirect and the choice for an optimal "gold standard" is not completely clear [373]. However, DEXA method has been regarded as a "gold standard" [374]. It is practicable and provides reproducible measurements of body components, i.e. fat mass, fat-free mass, bone-mineral mass. Furthermore, the validity of DEXA is high among most populations; errors have only been reported in younger and older populations [373]. Even though the radiation dose is low, further disadvantages of the DEXA method are its costs and trained radiology personnel are needed to operate. Another disadvantage exists especially with regard to obese participants, because they may exceed the maximum scan widths of the DEXA machine. If so, participants need to be "mummy wrapped" using thin sheets if Velcro straps were not sufficient, with arms placed in a lateral position to reduce participants width in order to get a result [324]. Skinfold measurements would not be an alternative to estimate %BF in obese adolescents, because they only poorly predict total fat mass if compared to DEXA [375].

Furthermore, it has been shown that among overweight and obese children, the DEXA method produced similar estimations for %BF as air-displacement-plethysmography (using a Bod Pod) and total body water (determined by deuterium oxide (^2H_2O) dilution using saliva

samples) compared to the four-compartment model [376], making DEXA an appropriate method for the RESIST study.

Within the DONALD study it is important to use a method that is applicable on an annual measurement basis and hence the DEXA method would not be an option. As already discussed in the original article (see OA1), hydrostatic weighing would be more precise method to estimate %BF [377]. This method is, however, not feasible for epidemiological studies or clinical trials such as those on which this thesis is based on. Even though the skinfold technique has been controversially discussed, it is probably the most widely used method in epidemiological studies, providing a direct measure of %BF [373]. Skinfold measurements are preferred within an epidemiological setting due to its low costs, but again, trained personnel are needed. To ensure quality of data within the DONALD study, the three study nurses undergo an annual quality control. More details on this are given in the original article (OA1, appendix 1; *Methods – Anthropometric measurements*). Nevertheless, one major limitation is that only subcutaneous fat is measurable by callipers, hence not all metabolically relevant fat, i.e. visceral fat, can be assessed.

BIA could be considered another alternative method feasible within a setting of a cohort study or clinical trial, because it is of simple practice, quick and safe [373]. However, relatively recent findings suggest that BIA is mainly useful because it includes height and weight within the equations to estimate fat and lean mass. The measurement of impedance itself adds only little to the final result and sometimes random error only [378]. Therefore, BIA does not seem to be an actual alternative within epidemiological studies [373].

Looking at these alternatives the DEXA method as well as skinfold measurements are the best methods practicable and available to be used in the RESIST and DONALD study, respectively.

Blood measurements

Because of the open cohort design of the DONALD study, many participants have not yet reached young adulthood and of those who did, to date, only one blood sample was available. Therefore, as it was discussed in the original article (OA4, appendix 4); the analysis was based on this single measurement of the GH-IGF axis in younger adulthood to represent long-term circulating levels. Nevertheless, an advantage of the analytics was that each sample was measured twice to obtain all parameters of the GH-IGF axis.

Epidemiological studies usually rely on single biological specimen (e.g. blood sample) from each participant, which is both for cost and logistical reasons [379, 380]. It could be argued

that repeated measurements of IGF-I and its binding proteins might more accurately reflect circulating levels [380]. However, in the case of the GH-IGF axis this may not be a problem as IGF-I values from repeated measurements (mean time between measurements was 42 days (SD 4.8)) were found to have a low intra-individual variation [381] and serum measurements of IGF-I and its binding proteins have been found to be quite representative of serum concentrations over longer time periods. In a subset of 76 participants of the New York University Women's Health Study correlations between repeated measurements (lag-time between the baseline and second visit ranged from 11-65 months, the median lag-time was 14 months) of IGF-I, IGFBP-3 and IGFBP-1 were strong, albeit weaker for IGFBP-2 (n=68) [382, 383].

In the RESIST study an OGTT was used to calculate the ISI, which was the primary outcome. According to Yeckel et al the ISI is a good and reliable method to assess whole body insulin sensitivity among obese children and adolescents. In fact, the ISI represents a good estimate of clamp-derived insulin sensitivity (r=0.78, p<0.0005) [384].

Lifestyle and parental characteristics

One problem of epidemiological studies is that covariates are often imperfectly measured or unmeasured. Of all variables obtainable for our analyses (e.g. early life and socioeconomic factors) a drawback was that only a relatively crude measure of physical activity was available (time spent outdoors, active, moderately active or inactive). Including this physical activity measure in the models did not change the results (OA1, OA3, and OA4). Since 2004, physical activity is assessed using a detailed questionnaire, but this data was only available for 35% (OA1 and OA3) and 37% (OA4) of all DONALD participants included in the analyses and was hence not used as covariate. Since 2013, physical activity is assessed with accelerometers which will be available in the future. In addition, sample sizes, which may be small, restrict the number of covariates. With the use of "too" many covariates the model will lack precision and will be unreliable to validly examine an association [385]. Overall, residual confounding may remain which cannot be controlled for. Adolescence in general is a period of change and there exist many potential influencing factors, such as socio-environmental, life style or psychological factors, which cannot be accounted for and may therefore confound when the relations between dietary exposures and outcomes are examined. The DONALD sample is, however, relatively homogenous, which might reduce vulnerability to residual confounding.

The RESIST trial has a relatively small sample size, which is not unusual for clinical trials, and owed to the fact that RESIST includes a high risk study population, i.e. obese adolescents with features of insulin resistance and/or prediabetes. Not only recruitment, as mentioned before, but also data assessment can be challenging and missing data may occur. Within the analysis only little adjustment was conceivable and it was for example not possible to adjust for physical activity or metformin treatment (compliance) as this data were not available for this analysis. With regards to metformin, this may not be a problem since all participants received a standard dosage (initial dose was 250 mg twice a day; after the first two weeks this was increased to a final dose of 500 mg twice a day). Regarding the family history, RESIST was relatively homogenous, as the majority of parents were overweight, obese or had a history of type 2 diabetes.

6.3   Public health considerations

The findings of this thesis suggest that a higher dietary insulin demand is potentially unfavourable for the development of body composition among healthy individuals and weight loss among obese adolescents. Furthermore, higher pubertal animal protein intake may be related to higher lean body mass and an up-regulation of the GH-IGF axis which persists until adulthood.

The question remains what the implications of a higher insulin demand and higher protein intake are. Theoretically, a higher insulin demand might be driven by a higher protein intake. However, particularly a co-ingestion of protein rich foods with carbohydrates was found to be an efficient insulin secretagogue in type 2 diabetic subjects allowing an improved response to ingested carbohydrates [16, 72, 386]. The specific stimulation of disproportionally high insulin secretion may reduce the glucose response of carbohydrate rich foods, thus postprandial glucose spikes may not occur. Possibly, in order to lower postprandial blood glucose levels, the specific stimulation of disproportionally high insulin responses by protein rich foods might postpone the need for medication of pre-diabetic individuals or support treatment of type 2 diabetic patients. On the other hand, higher postprandial glycaemic and insulinaemic excursions and greater demands on β-cell function could promote β-cell failure and a more rapid development of type 2 diabetes [56, 273]. A new study may shed light on this issue: The PREVention of diabetes through lifestyle Intervention and population studies in Europe and around the World (PREVIEW) study. It is a multicentre, clinical randomized intervention of 3 years duration including pre-diabetic participants of all ages and will compare two different diets (moderate-protein, moderate-GI or high-protein, low-GI) on

weight loss and type 2 diabetes development. The diet is based on findings from the DIOGenes study, which revealed that a high protein and low-GI diet was the best diet for weight loss maintenance among overweight adults [24] and prevention of obesity among children and adolescents [25]. When protein rich foods are ingested with carbohydrates higher insulin responses may however reduce postprandial glucose spikes. Overall, an increase of dietary protein intake may actually reduce the dietary insulin demand, especially when substituting insulin demanding carbohydrates for less insulin demanding proteins such as non-dairy proteins.

Findings also suggest a potential beneficial relation of higher animal and meat protein intakes and an increase in adult fat-free mass, which in turn would lead to a higher energy expenditure [164] and may prevent obesity. This has been discussed earlier in more detail, stressing the importance to distinguish between fat and lean body mass in observational studies and clinical trials (see chapter 6.1.2). But besides a potential favourable effect on body composition, higher animal and meat protein intakes may increase the risk of other chronic diseases. Increased intakes of animal protein [350] and red meat [387, 388] were prospectively related to an increased diabetes risk. In addition, Pan et al recently showed that red meat consumption was prospectively related to an increased risk of cardiovascular disease and cancer mortality [389]. The authors also estimated that a daily replacement of one serving of red meat with one serving of other foods, including fish, poultry, nuts, legumes, low-fat dairy, and whole grains, was associated with a 7% to 19% lower mortality risk [389], suggesting that a substitution of red meat would indeed be beneficial. In general, it is difficult to only consider a single nutrient or food such as animal protein or red meat, respectively, since this approach may be inadequate to unravel interactions with dietary-lifestyle pattern existing under free-living conditions. For instance, among adolescents a Western diet pattern has been identified, which was characterised by high intakes of processed and red meats, refined grains, French Fries, sweets and desserts, and sugar sweetened beverages [390]. This is considered an unhealthy diet and thus red meat might be a marker of an overall unhealthy lifestyle and contribute to adverse health effects. Hence, simply avoiding the consumption of animal (meat) protein in order to prevent the development of future chronic diseases may be too simplistic, since not only one nutrient, but the whole diet needs to be considered. It is of note, that the proportion of dietary protein intake in the highest tertile of animal protein intake was 14.4%En for females and 14.6%En for males and thus within German/European dietary recommendations [391, 392], showing that the habitually high animal (meat) protein observed in the DONALD population was still within the range of dietary guidelines. It is however not

clear for what an increased protein intake would be substituted. A moderate increase of (animal) protein intake may be favourable if it is substituted for unfavourable carbohydrates, whereas a substitution for favourable carbohydrates or fat may not be beneficial. It appears wise not to increase animal (meat) protein intake to a large extent as this would also increase the environmental burden [393] and may confer risks indicated by observational studies. Another aspect in this thesis was that higher animal (meat) protein intakes may upregulate the GH-IGF axis, but it remains to be elucidated whether this reflects a physiological adaptation or whether these associations indicate higher or lower risks of future diseases (see also OA4). In this thesis, no associations were found between plant protein intake and either body composition or the GH-IGF axis. Therefore, it can be speculated that increases in protein intake due to an increased plant protein intake may not result in adverse health effects. Overall, it is difficult to draw a final public health conclusion, however, if considering the whole diet a lower dietary insulin demand and a moderately higher protein intake may offer some benefits.

7. CONCLUSIONS AND PERSPECTIVES

The results presented in this thesis indicate that a lower dietary insulin demand and a higher dietary protein intake may be favourably related to body composition. In the view of a high obesity prevalence among children and adolescents as well as adults, preventive strategies starting early in life are urgently needed. Although these findings need to be confirmed in other populations, it seems prudent and conceivable that a reduction of the insulin demand of the diet resembles a safe and potentially promising dietary alternative. Besides a possible exchange of carbohydrate rich foods with a high FII for carbohydrate rich foods with a lower FII, this may also include a modest increase in the consumption of protein rich foods and hence, a moderate increase in dietary protein intake. This thesis suggests that moderate increases of dietary protein intake might not be detrimental, nonetheless with increased intakes the question of protein quality should be considered. Moreover, the results included in this thesis revealed that among women, a higher pubertal animal protein intake may induce an up-regulation of the adult GH-IGF-I axis. By contrast, inverse associations between higher animal protein intakes in early life and IGF-I concentrations among adult males support the idea that habitually higher animal protein intakes in this period may trigger an early programming of the GH-IGF axis. Yet, it has not been elucidated whether this reflects a physiological adaptation or higher or lower risks of future diseases.

Future studies are needed to confirm the specific relevance of the dietary insulin demand (during adolescence) for obesity risk and weight loss. In general, studies examining body composition should use indicators for fat mass and fat-free mass in addition to BMI. Furthermore, it would be interesting to know what the impact of an increase in plant protein consumption would be, since the results of this thesis do not suggest any adverse relation between plant protein intake and either body composition or the GH-IGF axis. Therefore, studies are required to disentangle differences in protein quality and to determine the relevance of plant protein intake for body composition as well as the GH-IGF axis. Also, a comprehensive appraisal of the relevance of dietary protein would need to differentiate between protein-carbohydrate-exchanges, e.g. substitution of carbohydrate rich foods having a high FII/GI for plant protein. With regards to the GH-IGF axis, it is not clear which mechanisms lie behind the observed programming and whether observed associations between dietary animal protein and the GH-IGF axis are a physiological adaptation, thus further research is needed.

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ORIGINAL ARTICLE

Prospective associations of dietary insulin demand, glycemic index, and glycemic load during puberty with body composition in young adulthood

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BACKGROUND: Puberty is a so-called critical period for overweight development and is characterized by physiological insulin resistance during mid-puberty. This study addressed the hypothesis that habitual consumption of a diet inducing higher levels of postprandial glycemia or insulinemia during puberty may have an unfavorable effect on the body composition in young adulthood.
METHODS: Multivariate regression analysis was performed on 262 participants of the Dortmund Nutritional and Anthropometric Longitudinally Designed Study with at least two 3-day weighed dietary records during puberty (baseline: girls 9–14 years; boys 10–15 years) and anthropometric measurements in young adulthood (18–25 years). A published dietary glycemic index was assigned to each carbohydrate-containing food. Similarly, each food was assigned a food insulin index (insulinemic response to a 1 MJ portion of food relative to 1 MJ of glucose) using 121 values measured at Sydney University.
RESULTS: Dietary glycemic index or glycemic load during puberty was not related to body composition in young adulthood. In contrast, a higher dietary insulin index and a higher dietary insulin load during puberty were associated with higher levels of percentage of body fat (%BF) in young adulthood, even after adjustment for early life, socioeconomic and nutritional factors; %BF in energy-adjusted tertiles of dietary insulin index were 22.9 (95% confidence intervals (CI): 21.6, 24.1), 24.5 (23.2, 25.7), 24.7 (23.5, 25.9) %, $P_{for\ trend} = 0.01$; %BF in energy-adjusted tertiles of dietary insulin load were 22.8 (95% CI: 21.5, 24.0), 24.5 (23.2, 25.7), 24.8 (23.6, 26.0) %, $P_{for\ trend} = 0.01$. Adjustment for baseline %BF attenuated these relationships ($P_{for\ trend} = 0.1$ and $= 0.08$, respectively). Dietary insulin demand was not related to body mass index.
CONCLUSION: This study suggests a prospective adverse influence of dietary insulin demand during puberty on %BF in young adulthood. Postprandial increases in insulinemia rather than increases in glycemia appear to be implicated in an unfavorable development of body composition.

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Keywords: dietary insulin index; dietary insulin demand; dietary glycemic index; dietary glycemic load; body composition; body fat

INTRODUCTION

Over the previous years, the relevance of the dietary glycemic index (GI) for the development of obesity has been controversially debated. Among adults, prospective cohort studies suggest a role of the dietary GI for body composition.[1-4] However, similar associations have not been observed among healthy children and adolescents[5,6] or overweight Latino adolescents.[7]

Intervention studies in overweight and obese adults suggest a specific efficacy of low-GI weight-loss diets[8,9] for persons with already increased insulin secretion levels.[9] Puberty is a so-called 'critical period' for overweight development, which is characterized by physiological insulin resistance and changes in levels of various hormones, including insulin-like growth factor (IGF)-1, growth hormones as well as sex steroids.[10] In fact, IGF-1 levels rise steeply during puberty and peak before the end of puberty, whereas the development of the insulin sensitivity follows the reverse course.[11,12]

It is possible that postprandial glycemia and insulinemia are relevant targets during puberty so as to prevent the development of an unfavorable body composition. Mechanisms linking the habitual consumption of high-GI foods to body composition include reduced satiety signaling, as fully gelatinized starches in high-GI foods do not reach the lower parts of the ileum, and enhanced carbohydrate oxidation and decreased fat oxidation in response to habitual postprandial glycemia and insulinemia.[13] In addition, reactive hypoglycemia in the late postprandial phase has been proposed to induce hunger and higher voluntary energy intakes.[14] Counter-regulatory hormone responses following this reactive hypoglycemia may have proteolytic effects, favoring the loss of lean body mass and a reduction of resting energy expenditure.[13] Finally, elevated IGF-1 levels may predispose to obesity later in life,[15] and the GI of a meal has been found to acutely affect the IGF-1 axis.[16]

As high-GI foods influence both blood glucose and insulin levels, it is not clear which of these postprandial changes is potentially more relevant for an unfavorable development of body composition. Insulin secretion is also stimulated by dietary protein and, moreover, dietary protein and fat may both act synergistically with carbohydrates, raising insulin levels and reducing postprandial glycemia.[17-19] The food insulin index (FII) compares the

postprandial insulin response to any food relative to a reference food (glucose) and also, unlike the GI, considers foods with no or low amounts of carbohydrates.[20]

This study addressed the hypothesis that habitual consumption of a diet inducing higher levels of postprandial glycemia or insulinemia during puberty may have an unfavorable effect on body composition in young adulthood.

METHODS

Study population

The present study was ancillary to the Dortmund Nutritional and Anthropometric Longitudinally Designed Study (DONALD Study), an ongoing, open cohort study conducted at the Research Institute of Child Nutrition in Dortmund, Germany. Details on this study have been described elsewhere.[21]

Briefly, as the recruitment began in 1985, detailed data on diet, growth, development, and metabolism between infancy and adulthood have been collected from >1300 healthy children. Every year, an average of 50 infants are newly recruited and first examined at the age of 3 months. Each child returns for three more visits in the first year, two in the second and then once annually until adulthood. The study was approved by the Ethics Committee of the University of Bonn, and all examinations are performed with parental consent.

The children who were initially recruited for the DONALD Study differed considerably in age. Because of the open cohort design, many children have not yet reached young adulthood. In total, 394 subjects were aged 18 years or older by the time of this analysis. They were term (37–42 week gestation) singletons with a birth weight $\geqslant$2500 g and had at least one anthropometric measurement in young adulthood. As we were interested in the long term-relevance of dietary parameters during adolescence for adult body composition, we regressed dietary intake on the last anthropometric measurement available during young adulthood ($\geqslant$18 and $\leqslant$25 years of age, mean age = 20.3 years). Of these, 308 participants had provided at least two 3-day weighed dietary records at baseline (puberty was defined by chronological age: girls 9–14 years, boys 10–15 years), allowing the estimation of habitual dietary intake during puberty. Participants who were identified to consistently underreport their energy intake (that is, all food records were implausible or they had provided more implausible than plausible food records) were excluded from the study ($n = 23$). A 3-day weighed dietary record was considered plausible when the total recorded energy intake was adequate in relation to the basal metabolic rate (estimated from the Schofield equations[22]) using modified cut-offs from Goldberg *et al.*[22,23] Overall, 1379 records were included (2–7 records per participant). Furthermore, participants had to have anthropometric data available at baseline and information on relevant covariates such as early life (for example, breast feeding) and socioeconomic factors (for example, maternal overweight). This resulted in a final sample of 262 participants (53.6% female, 46.4% male).

Anthropometric measurements

Participants are measured at each visit according to standard procedures,[24] dressed in underwear only and barefoot. From the age of 2 years onward, standing height is measured to the nearest 0.1 cm using a digital stadiometer (Harpenden Ltd., Crymych, UK). Body weight is measured to the nearest 100 g using an electronic scale (Seca 753E; Seca Weighing and Measuring Systems, Hamburg, Germany). Skinfold thicknesses are measured from the age of 6 months onward at four different sites (supra-iliacal, subscapular, biceps, triceps) on the right side of the body to the nearest 0.1 mm using a Holtain caliper (Holtain Ltd., Crosswell, United Kingdom). Since 2005, waist circumference is also routinely measured according to World Health Organization recommendations at the midpoint between the lower rib margin and the iliac crest.[25] The three trained nurses who perform the measurements undergo an annual quality control, conducted in six to eight healthy young adult volunteers. Average inter- and intra-individual variation coefficients obtained in the last 6 years (2005–2010) were 0.7 and 1.8% for waist circumference, 7.9 and 12.7% for biceps,

5.4 and 6.2% for triceps, 5.2 and 7.8% for subscapular, and 7.5 and 9.1% for supra-iliacal skinfolds.

Anthropometric calculations

Regarding body mass index (BMI, $kg\,m^{-2}$) in puberty, sex- and age-independent standard deviation scores were calculated using the German reference curves for BMI.[26] Percentage body fat (%BF) was derived using equations of Slaughter *et al.*[27] for pubescent children, which consider triceps and subscapular skinfolds. Overweight during puberty was defined according to values proposed by the International Obesity Task Force, which correspond to an adult BMI of 25 $kg\,m^{-2}$.[28] The reference values for %BF published by McCarthy *et al.*[29] were used to determine pubertal participants with excess body fatness, that is, %BF above the 85th percentile.[29]

Regarding anthropometric data in young adulthood, BMI was calculated and %BF was estimated from skinfolds using Durnin and Womersley equations,[30] which are based on triceps, biceps, scapular and iliacal skinfolds.

Nutritional assessment

Food consumption in the DONALD Study is assessed annually using 3-day weighed dietary records. All foods and beverages consumed are weighed and recorded, as well as leftovers, to the nearest 1 g over 3 days using electronic food scales (initially Soehnle Digita 8000; Leifheit SG, Nassau; Germany; now WEDO digi 2000; Werner Dorsch Gmbh, Muenster/Dieburg, Germany). For this analysis, dietary variables were calculated as individual means of the 3-day weighed dietary records using LEBTAB,[31] the in-house database, which is continuously updated to include all recorded food items. LEBTAB is based on the German standard food tables[32] and data obtained from commercial food products. Currently, LEBTAB contains more than 13 100 entries, including additives, supplements and medicine, that is, 1207 basic food items and 10 832 composite foods.

To better describe the habitual dietary intake during puberty, an individual average intake was calculated from at least two records during puberty.

Dietary GI and insulin index

Dietary GI is defined as the incremental area under the curve of glucose response following the intake of 50 g of carbohydrate from a test food as compared with area under the curve of glucose response induced by the same amount of carbohydrate ingested as glucose in 5–10 separate individuals.[33] A published GI value[34] was assigned to each carbohydrate-containing food recorded in the dietary records (based on glucose as the reference food) according to a standardized procedure.[35] The carbohydrate content (in grams) of each consumed food was then multiplied by the food's GI to obtain its glycemic load (GL). The sum of these GL values for each subject corresponds to the total daily GL. The overall GI is obtained by dividing the total daily GL by the total daily carbohydrate intake.

The FII is defined as the insulinemic response (area under the curve) following the intake of 1000 kJ of a food relative to the insulinemic response to glucose that is, the reference food (FII = 100).[20] Foods originally tested against a white-bread standard were converted to the glucose standard by a conversion factor of 0.73. For the present analysis, 121 FII values measured at Human Nutrition Unit School of Molecular and Microbial Biosciences University of Sydney, Australia in groups of 10 individuals[36] were available to assign a FII value to each food recorded in the dietary records according to a standardized procedure similar to that established for GI assignment (Figure 1). The dietary GL was the principal consideration when matching foods rich in carbohydrates with an available FII, as it is the best predictor of FII.[20,36] The protein content was used as a guide to find the best match when carbohydrate content was low. A published FII or a close match was available for 36% of the foods (steps 1 and 2), a weighted mean was calculated for another 33% (step 4) and 18% of the foods were assigned the mean FII of the respective food group (step 3). For 11% of the foods the FII value was assigned
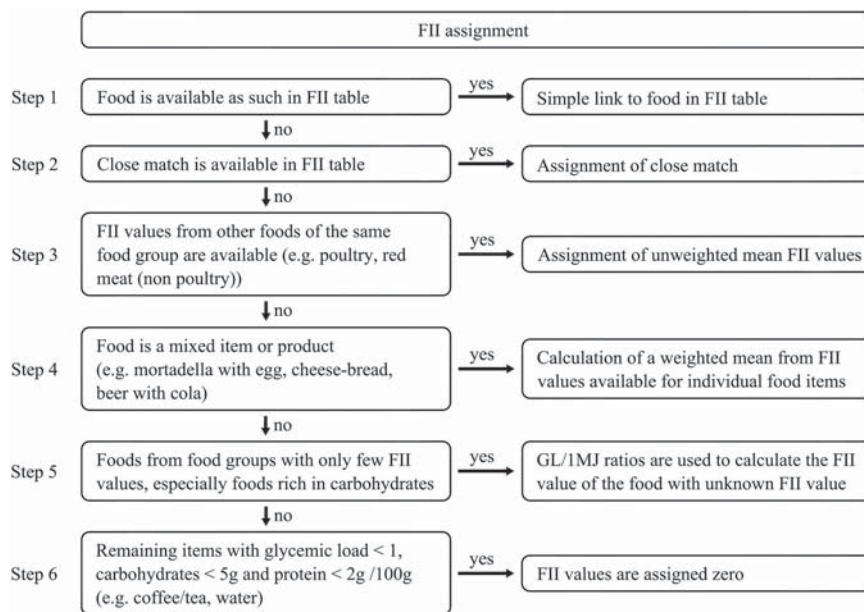
Figure 1. Flowchart for the assignment of FII values to food items recorded in the 3-day weighed dietary records (FII values measured at Human Nutrition Unit School of Molecular and Microbial Biosciences University of Sydney, Australia).

zero (step 6) and the GL ratio was used to calculate the FII of 3% foods (for example, $FII_{sucrose} = GL_{sucrose}/GL_{glucose} \times FII_{glucose}$; step 5). The average dietary insulin load was calculated by summing the product of FII, energy content and consumption frequency over all recorded food items in the 3-day dietary records. The average dietary insulin index was calculated by dividing the insulin load by total energy intake.[37]

Potentially confounding factors

On their child's admission to the study, parents are interviewed by the study pediatrician, and weighed and measured by the study nurses using the same equipment as for children from 2 years onward. Information on the child's birth characteristics are abstracted from the 'Mutterpass', a standardized document given to all pregnant women in Germany. The duration of full breastfeeding (no solid foods and no liquids other than breast milk, tea or water) is inquired by the pediatricians at the first visits until complementary feeding is initiated. For this analysis the following characteristics were considered: breastfeeding status (ever fully breastfed (yes/no) was defined as fully breastfed >2 weeks), maternal overweight status (BMI ⩾25 kg m^{-2}), high maternal educational status (⩾12 years of schooling) and smoking in the household (yes/no).

Statistical analysis

To analyze the potential relation of dietary insulin index, insulin load, GI and GL during puberty with body composition in young adulthood, the distribution of these dietary variables was grouped into tertiles (T1–T3). Tests for differences were performed among the tertiles of dietary insulin index, insulin load, GI and GL using ANOVA for normally distributed continuous variables, Kruskal–Wallis test for non-normally distributed continuous variables and χ^2-test for categorical variables. Analysis of the association between diet during puberty and body composition in young adulthood was performed by multiple linear regression analysis. As BMI was not normally distributed it was log-transformed before the analysis. Each potential confounder was initially considered separately and included if it modified the respective association substantially. Thus, sex was retained in the basic model (model A). In a further step, we also adjusted for early life (breastfeeding) and socioeconomic factors (maternal overweight) as well as other nutritional factors (model B). In a final model, we controlled for confounding by body composition at baseline (model C). All dietary variables except dietary insulin index and GI were energy adjusted

using the residual method.[38] To account for age-dependent changes in intake levels all variables were standardized by age group and sex (mean = 0, s.d. = 1).[6]

The adjusted means (that is, least-squares means predicted by the model when the other variables were held at their mean values) are presented with their 95% confidence interval by tertiles. *P*-value <0.05 was considered as statistically significant. All statistical analyses were carried out using SAS procedures (version 9.1.3, SAS Institute, Cary, NC, USA).

RESULTS

Subjects who were excluded from the study sample (*n* = 132) did not differ in sex, birth weight or length, gestational age, BMI and %BF in young adulthood from those who were included (*n* = 262) (data not shown).

Baseline characteristics of the 262 healthy participants did not differ across tertiles of GI and GL. However, subjects with a diet in the lowest tertile of insulin index and insulin load were less likely to be overweight at baseline and those in the lowest tertile of insulin load tended to have lower levels of %BF at baseline. Furthermore, participants in the lowest tertile of the dietary insulin load were less likely to have had mothers with a high level of education (Table 1). Mean BMI–standard deviation scores during puberty were close to zero, indicating that the BMI values at baseline were comparable to the German reference population.

Participants in the highest dietary insulin index and insulin load tertile had lower of total and saturated fat, total and animal protein, but higher intakes of vegetable protein, carbohydrate, and added sugar (% of total energy, %E) as well as higher dietary GI and GL compared with participants in the lowest dietary insulin index and insulin load tertile (Table 2). Comparable differences were seen across tertiles of GL. A higher dietary GI was related to lower intakes of total and animal protein as well as fiber, and higher added sugar intake (%E), a higher dietary insulin index and a higher GL.

Overall, dietary insulin index, insulin load, GI and GL during puberty were not related to BMI in young adulthood, (Table 3) and dietary GI and GL during puberty were not related to %BF in young adulthood (Table 4). However, a higher dietary insulin index

Table 1. Demographic, anthropometric, birth, and socioeconomic characteristics by energy-adjusted tertiles of dietary insulin index, insulin load, GI, and GL at baseline (n = 262), DONALD Study, Germany

	Subjects	Dietary insulin index at baseline				Dietary insulin load at baseline				Dietary GI at baseline				Dietary GL at baseline			
	n	T1	T2	T3	P value[a]	T1	T2	T3	P value[a]	T1	T2	T3	P value[a]	T1	T2	T3	P value[a]
Female (n (%))	262	46 (53.5)	47 (53.4)	47 (53.4)	>0.9	46 (53.5)	47 (53.4)	47 (53.4)	>0.9	46 (53.5)	47 (53.4)	47 (53.4)	>0.9	46 (53.5)	47 (53.4)	47 (53.4)	>0.9
Age (years)[b]	262	9.8	9.9	9.3	0.7	9.8	9.9	9.3	0.6	9.9	9.5	9.2	0.7	9.8	9.8	9.6	>0.9
Weight (kg)[b]	262	32.3	34.6	34.5	0.5	33.1	33.0	35.0	0.5	34.9	33.1	32.7	0.3	33.5	34.9	33.4	0.7
Height (m)[b]	262	139.7	142.1	142.2	0.6	139.9	141.3	142.5	0.8	142.0	140.0	142.1	0.2	141.0	142.1	141.1	0.7
BMI–SDS	262	-0.12	-0.02	0.06	0.4	-0.08	-0.10	0.10	0.3	0.10	-0.03	-0.15	0.2	0.06	-0.05	-0.09	0.5
BMI (kg m^{-2})[b]	262	16.6	16.7	16.8	0.6	16.9	16.5	16.9	0.3	17.2	16.7	16.1	0.2	17.0	16.7	16.5	0.5
Overweight (n (%))[c]	262	6 (7.0)	12 (13.6)	16 (18.2)	0.09	6 (7.0)	12 (13.6)	16 (18.2)	0.09	11 (12.8)	13 (14.8)	10 (11.4)	0.8	10 (11.6)	11 (12.5)	13 (14.8)	0.8
Body fatness (%)[b,d]	262	15.1	16.3	17.4	0.1	15.1	16.3	17.5	0.09	15.4	16.4	16.0	0.9	16.3	15.9	16.0	0.7
Excess body fat (n (%))[e]	262	11 (12.8)	15 (17.1)	19 (21.6)	0.3	12 (14.0)	14 (15.9)	19 (21.6)	0.4	14 (16.3)	15 (17.1)	16 (18.2)	0.9	15 (17.4)	12 (13.6)	18 (20.5)	0.5
Birth weight (g)	262	3443	3429	3542	0.2	3458	3423	3533	0.2	3506	3473	3436	0.6	3477	3481	3456	0.9
Birth length (cm)[b]	262	51.5	52.0	52.0	0.5	52.0	51.0	52.0	0.7	52.0	52.0	51.0	0.5	52.0	52.0	52.0	0.8
Pregnancy duration (weeks)[b]	262	40.0	40.0	40.0	0.6	40.0	40.0	40.0	0.2	40.0	40.0	40.0	0.5	40.0	40.0	40.0	0.2
Breast feeding (>2weeks (n (%))[f]	262	58 (67.4)	66 (75.0)	61 (69.3)	0.5	56 (65.1)	69 (78.4)	60 (68.2)	0.1	62 (72.1)	56 (63.6)	67 (76.1)	0.2	60 (69.8)	61 (69.3)	64 (72.7)	0.9
Maternal overweight (n (%))[g]	262	22 (25.6)	31 (35.2)	31 (35.2)	0.3	22 (25.6)	31 (35.2)	31 (35.2)	0.3	24 (27.9)	27 (30.7)	33 (37.5)	0.4	23 (26.7)	30 (34.1)	31 (35.2)	0.4
Maternal education (n (%))[h]	260	30 (35.3)	44 (50.0)	41 (47.1)	0.1	28 (32.9)	47 (54.0)	40 (45.5)	0.02	40 (46.5)	37 (42.5)	38 (43.7)	0.9	30 (35.3)	44 (50.6)	41 (46.6)	0.1
Smoking in the household (n (%))	208	25 (34.7)	22 (31.9)	23 (34.3)	0.9	25 (34.2)	20 (28.6)	25 (37.3)	0.5	15 (22.7)	29 (40.3)	26 (37.1)	0.07	23 (31.9)	23 (32.4)	24 (36.9)	0.8

Abbreviations: BMI, body mass index; GI, glycemic index; GL, glycemic load; SDS, standard deviation scores; T, tertile. [a]Significant differences between the tertiles were tested using analysis of variance for normally distributed continuous variables, Kruskal–Wallis test for not normally distributed continuous variables and χ^2-test for categorical variables. Values are means unless indicated as medians[b] or otherwise. [c]Derived from the age- and sex-specific cut-points proposed by the International Obesity Task Force, which are linked to the adult cut-off point of a BMI of 25 kg m^{-2}.[28] [d]Calculated according to Slaughter et al.[27] [e]Derived from age-specific cut-points proposed by McCarthy et al.,[29] the 85th percentile of body fat was used as cut-off for excess of body fat. [f]Breast feeding categories: ≤2 weeks, >2 weeks of full breastfeeding. [g]Maternal BMI ≥25 kg m^{-2}. [h]School education for at least 12 years.

Table 2. Baseline nutritional data by energy-adjusted tertiles of dietary insulin index, insulin load, GI, and GL at baseline ($n = 262$), DONALD Study, Germany

Subjects	Dietary insulin index at baseline				Dietary insulin load at baseline				Dietary GI at baseline				Dietary GL at baseline			
	T1	T2	T3	P value[a]	T1	T2	T3	P value[a]	T1	T2	T3	P value[a]	T1	T2	T3	P value[a]
All (n)	86	88	88		86	88	88		86	88	88		86	88	88	
Total energy (MJ per day)[b]	7.9	7.9	7.9	>0.9	7.9	7.8	7.9	0.8	7.9	7.6	8.1	0.7	7.9	7.5	8.0	0.5
Fat (% of energy)	38.7	35.2	33.2	<0.0001	38.7	35.3	33.2	<0.0001	35.7	36.0	35.4	0.6	39.0	35.6	32.6	<0.0001
Saturated fatty acid (% of energy)	16.9	15.4	14.6	<0.0001	16.9	15.4	14.6	<0.0001	15.9	15.8	15.3	0.2	17.1	15.7	14.1	<0.0001
Protein (% of energy)	13.8	12.9	12.4	<0.001	13.8	12.9	12.5	<0.0001	13.7	12.9	12.5	<0.0001	13.9	13.0	12.3	<0.0001
Animal protein (% of energy)	9.2	8.1	7.4	<0.0001	9.2	8.0	7.5	<0.0001	9.0	8.0	7.7	<0.0001	9.3	8.0	7.4	<0.0001
Vegetable protein (% of energy)	4.6	4.9	5.0	0.001	4.6	4.9	5.0	0.0008	4.8	4.9	4.8	0.4	4.6	5.0	4.9	0.01
Consumers of alcohol (n (%))	7 (8.1)	8 (9.1)	7 (8.0)	>0.9	7 (8.1)	8 (9.1)	7 (8.0)	>0.9	13 (15.1)	7 (8.0)	2 (2.3)	0.009	7 (8.1)	8 (9.1)	7 (8.0)	>0.9
Carbohydrate (% of energy)	47.4	51.8	54.3	<0.0001	47.5	51.8	54.2	<0.0001	50.5	51.0	52.0	0.07	47.0	51.3	55.1	<0.0001
Added sugar (% of energy)	12.6	14.7	16.1	<0.0001	12.7	14.6	16.2	<0.0001	12.4	13.9	17.1	<0.0001	11.8	14.2	17.5	<0.0001
Dietary insulin index	39	42	45	<0.0001	39	42	45	<0.0001	41	42	43	<0.0001	40	42	44	<0.0001
Dietary insulin load	319	338	359	0.0002	321	333	362	<0.0001	332	334	350	0.1	322	336	358	0.0006
Dietary GI	55.0	56.1	56.7	<0.0001	55.1	56.1	56.7	<0.0001	53.3	56.0	58.5	<0.0001	54.4	56.1	57.3	<0.0001
Dietary GL (g)[b]	124.4	133.0	143.4	<0.0001	123.8	132.1	144.9	<0.0001	126.6	131.9	144.6	0.0001	122.7	131.6	149.5	<0.0001
Fiber (g)[b]	18.9	19.2	18.4	0.9	18.9	19.1	18.4	0.9	20.4	18.5	17.5	0.0006	19.3	19.0	18.3	0.6

Abbreviations: GI, glycemic index; GL, glycemic load; T, tertile. [a]Significant differences between the tertiles were tested using analysis of variance for normally distributed continuous variables, Kruskal–Wallis test for not normally distributed continuous variables and χ^2-test for categorical variables. Values are means unless indicated as medians[b] or otherwise.

Table 3. Relation of dietary insulin index, insulin load, GI, and GL at baseline to body mass index ($kg\,m^{-2}$) in young adulthood ($n = 262$), DONALD Study, Germany

	T1	T2	T3	P$_{for\ trend}$
Insulin index				
Model A	22.5 (21.9, 23.2)	22.5 (21.8, 23.2)	22.8 (22.2, 23.5)	0.5
Model B	23.1 (22.3, 23.8)	23.0 (22.3, 23.7)	23.3 (22.6, 24.0)	0.5
Model C	23.0 (22.4, 23.6)	22.7 (22.2, 23.3)	22.9 (22.4, 23.5)	0.8
Insulin load				
Model A	22.6 (21.9, 23.3)	22.3 (21.6, 22.9)	23.0 (22.3, 23.7)	0.5
Model B	23.1 (22.4, 23.8)	22.9 (22.2, 23.6)	23.4 (22.7, 24.1)	0.6
Model C	22.9 (22.3, 23.5)	22.7 (22.2, 23.3)	23.0 (22.4, 23.6)	0.9
GI				
Model A	22.8 (22.1, 23.5)	22.5 (21.8, 23.2)	22.5 (21.8, 23.2)	0.8
Model B	23.0 (22.3, 23.7)	23.0 (22.3, 23.7)	23.3 (22.6, 24.0)	0.4
Model C	22.8 (22.2, 23.4)	22.8 (22.2, 23.3)	23.1 (22.5, 23.7)	0.4
GL				
Model A	23.2 (22.5, 23.9)	22.6 (21.9, 23.3)	22.1 (21.4, 22.8)	0.3
Model B	23.3 (22.6, 24.1)	23.1 (22.5, 23.8)	22.8 (22.1, 23.5)	0.4
Model C	23.3 (22.7, 23.9)	22.9 (22.3, 23.5)	22.5 (21.9, 23.1)	0.6

Abbreviations: GI, glycemic index; GL, glycemic load; T, tertile. Values are means and 95% confidence interval. Model A: adjusted for sex. Model B: adjusted for sex, early life factors (breast feeding), socioeconomic factors (maternal overweight) and nutritional factors (insulin index: energy; insulin load: energy; GI: energy, fiber, protein; GL: energy, fiber, protein). Model C: Model B + adjustment for baseline (body mass index).

and insulin load during puberty was associated with a higher %BF in young adulthood, even after adjustment for early life, socioeconomic and nutritional factors (model B for insulin index and insulin load, both $P_{for\ trend} = 0.01$). Additional consideration of baseline %BF attenuated these relationships (model C, $P_{for\ trend} = 0.1$ for insulin index and $P_{for\ trend} = 0.08$ for insulin load). Model B did not include fiber as a covariate because it did not affect the associations between dietary insulin index or insulin load and body composition. Intakes of carbohydrate, protein or fat were not considered because those macronutrients contribute to the dietary insulin index and insulin load. However, as protein may also conduce higher lean mass,[39] we included this macronutrient as a covariate in a further step (data not shown) and observed a similar association between higher dietary insulin index and insulin load during puberty, and higher %BF in young adulthood (insulin index: $P_{for\ trend} = 0.0965$; insulin load: $P_{for\ trend} = 0.07$).

In an additional analysis we included carbohydrates and protein to address the effect of qualitative changes in dietary insulin index only, by holding the macronutrient intake constant, that is, the effect of substituting carbohydrate- and protein-rich foods of a high insulin demand for carbohydrate- and protein-rich foods with a low insulin demand on %BF in young adulthood. Using this qualitative approach, a higher dietary insulin index (Figure 2, Panel A) and a higher dietary insulin load (Figure 2, Panel B) were both related to a higher %BF in young adulthood even when controlling for baseline %BF ($P_{for\ trend} = 0.04$ and $P_{for\ trend} = 0.03$,

| | T1 | T2 | T3 | P_{for trend} |

Table 4. Relation of dietary insulin index, insulin load, GI, and GL at baseline to percentage body fat in young adulthood ($n = 262$), DONALD Study, Germany

	T1	T2	T3	$P_{for\ trend}$
Insulin index				
Model A	22.3	23.8	24.2	0.01
	(21.1, 23.4)	(22.7, 25.0)	(23.0, 25.3)	
Model B	22.9	24.5	24.7	0.01
	(21.6, 24.1)	(23.2, 25.7)	(23.5, 25.9)	
Model C	23.2	24.2	24.2	0.1
	(22.1, 24.3)	(23.1, 25.3)	(23.1, 25.3)	
Insulin load				
Model A	22.2	23.7	24.3	0.007
	(21.1, 23.4)	(22.5, 24.9)	(23.2, 25.5)	
Model B	22.8	24.5	24.8	0.01
	(21.5, 24.0)	(23.2, 25.7)	(23.6, 26.0)	
Model C	23.1	24.2	24.3	0.08
	(22.0, 24.2)	(23.1, 25.3)	(23.3, 25.4)	
GI				
Model A	23.3	23.5	23.5	0.9
	(22.1, 24.5)	(22.4, 24.7)	(22.3, 24.7)	
Model B	23.5	24.1	24.4	0.7
	(22.2, 24.9)	(22.9, 25.3)	(23.1, 25.6)	
Model C	23.7	24.0	24.0	> 0.9
	(22.5, 24.8)	(22.9, 25.1)	(22.9, 25.1)	
GL				
Model A	23.8	23.6	22.8	0.8
	(22.6, 25.0)	(22.5, 24.8)	(21.7, 24.0)	
Model B	24.3	24.3	23.5	0.4
	(23.0, 25.6)	(23.1, 25.5)	(22.2, 24.8)	
Model C	24.3	24.2	23.2	> 0.9
	(23.1, 25.5)	(23.1, 25.3)	(22.1, 24.3)	

Abbreviations: GI, glycemic index; GL, glycemic load; T, tertile. Values are means and 95% confidence intervals. Model A: adjusted for sex. Model B: adjusted for sex, early life factors (breast feeding), socioeconomic factors (maternal overweight) and nutritional factors (insulin index: energy; insulin load: energy; GI: energy, fiber, protein; GL: energy, fiber, protein). Model C: Model B + adjustment for baseline (percentage body fat).

Figure 2. Percentage body fat in young adulthood by energy-adjusted tertiles of dietary insulin index (II) (**a**) and insulin load (IL) (**b**) during puberty (baseline) for 262 subjects. Data are means (95% CI) adjusted for sex, early life factors (breast feeding), socioeconomic factors (maternal overweight), nutritional factors (energy, carbohydrates, protein) and percentage body fat at baseline. *P* for trend refers to the *P* value obtained in linear regression models with percentage body fat as continuous variable. T, tertile.

respectively). Similar results were obtained when adjusting for intakes of carbohydrates and fat, or intakes of protein and fat (data not shown).

Dietary insulin index, insulin load, GI and GL were not related to waist circumference, which was, however, available for a subsample of 196 participants only (data not shown).

We performed a number of additional analyses using

- the minimum number of two dietary records per subject only, randomly selecting two records for those participants who had provided more than two records ($n = 262$)
- the first anthropometric measurement in young adulthood as an outcome ($n = 262$)
- anthropometric measurements at the age of 18 years as an outcome ($n = 218$)

All approaches yielded similar results for the relationships of the dietary insulin index or insulin load to %BF (data not shown).

DISCUSSION
To the best of our knowledge, the present study provides new epidemiological evidence on a prospective relevance of dietary insulin demand during puberty for %BF in young adulthood
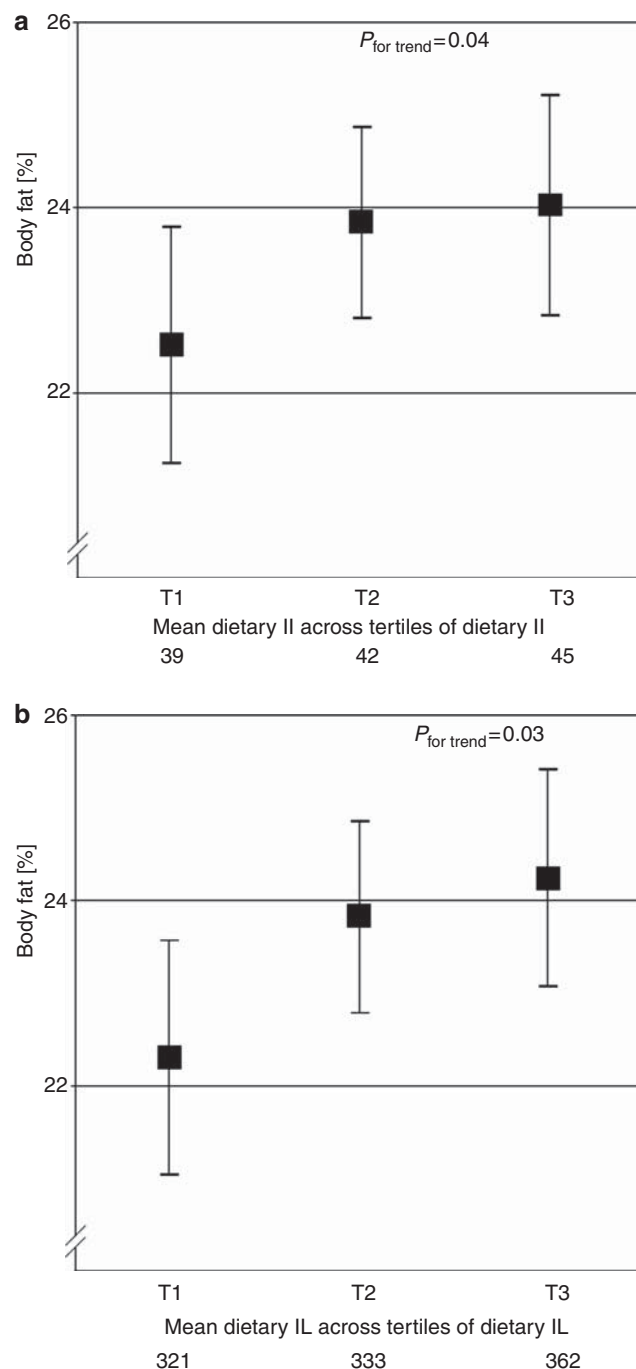
among a healthy free-living population. Although our data are purely observational and hence need to be interpreted cautiously, our study suggests that postprandial rises in insulinemia rather than glycemia may have adverse consequences for the development of body composition in early adulthood.

The mechanistic role of high postprandial insulin levels for a specific gain in %BF may be traced to the preferential direction of nutrients away from oxidation in muscle and toward storage in fat.[40] In line with this, Chaput *et al*.[41] reported that postprandial hyperinsulinemia at 30 min strongly predicted weight gain and change in waist circumference over 6 years in adults, especially among those consuming lower-fat diets. Furthermore, high insulin and low plasma glucagon levels may restrain hepatic glucose production and suppress lipolysis.[42] Thus, over a longer term, consistently high postprandial demand on the beta-cells may eventually reduce insulin sensitivity[14] and also promote the development of higher %BF.[43,44]

Another plausible mechanism by which a high dietary insulin index or insulin load (that is, insulin demand) may contribute to a higher %BF may work through cross-stimulation of both insulin and IGF-1 secretion.[45] *In vitro* studies using cultures of adipocyte precursor cells found a stimulatory effect of higher levels of IGF-1 on the proliferation of preadipocytes, which may therefore contribute to body-fat formation. Furthermore, IGF-1 stimulated the cellular glucose uptake in preadipocytes and adipocytes, increased lipogenesis and inhibited lipolysis in adipocytes.[46] We speculate that the physiological insulin resistance and the concurrent elevations of IGF-1 levels during puberty may work together to increase the susceptibility to postprandial insulinemic spikes and thus contribute to the development of high body fat.

In our view, it is plausible that we did not observe an association between dietary GI and body composition in our cohort of relatively lean subjects with physiological insulin resistance affecting peripheral tissues,[47] as a higher dietary GI may be of relevance primarily among persons who already respond with exaggerated insulin responses.[9] This may also explain why other studies reported associations between dietary GI and body composition mainly among overweight and less insulin-sensitive persons.[8,9] Conversely, we had expected to find at least a tendency for a comparable relation between dietary GL and body composition, as dietary GL has recently been identified as the best indirect predictor of the postprandial insulin response.[36] However, although the main contribution to the insulin responses arises from carbohydrate-rich foods, Bao *et al*.[36] reported dietary GL to explain only 46% of the observed variability in insulin responses, that is, foods with little or no carbohydrates and a higher protein and fat content make additional important contributions.

It could be argued that the association between dietary insulin demand and unfavorable body composition may be primarily attributable to one macronutrient only (for example, carbohydrates). However, our additional analysis adjusting for protein, carbohydrates and energy suggests that in particular substitutions of carbohydrate- and protein-rich foods with a higher insulin demand for carbohydrate- and protein-rich foods with a lower insulin demand are the relevant principle for the associations with body fat. In addition, further adjustment for protein enhanced the association between dietary insulin demand and body fat. This may reflect a bi-directional relevance of dietary protein, which may contribute to a higher lean body mass on the one hand[39] and a higher insulin secretion[17] or lower insulin clearance on the other hand.[48]

The relationship between a higher dietary insulin demand during puberty and a higher %BF in young adulthood was attenuated by the additional consideration of baseline %BF. While this confirms the prevailing long-term relevance of %BF already in childhood, we may have also corrected for earlier effects of dietary insulin demand on body composition. It may be that the dietary insulin demand has a more important role in adolescents who are overweight or have a higher %BF. In our sample we did not find a consistent interaction between overweight or excess body fat at baseline and dietary insulin demand concerning %BF in young

adulthood, but this may be attributable to the fact that our sample is comparatively healthy with lower prevalences of overweight or excess body fat.

Our study has several limitations. First, we applied the FII concept, developed to quantify the insulin response to foods – to estimate the dietary insulin demand. Hence, the limitations debated for the estimations of dietary GI[49–51] also apply to the estimation of dietary insulin demand. As the FII assignment was based only on 121 published FII values it must be considered crude, yet allowing a classification of foods in FII groups.[52] Second, %BF was estimated from skinfold thickness measurements, which are known to be more susceptible to measurement error than are specialized research-based techniques. Other more accurate methods to estimate %BF, such as hydrostatic weighing, may be preferable to estimate body fat,[53] but the skinfold equations of Durnin and Womersley[30] are feasible and agree, on average, very well with results from hydrostatic weighing.[53] Furthermore, measurements were conducted by trained and quality-monitored personnel, which has been shown to reduce intra- and inter-observer variability considerably,[54] as was the case in the present study. Third, the DONALD population has a relatively high socioeconomic status,[55] as reflected by the parental educational level. It is possible that the relative homogeneity of the healthy DONALD sample means that extremes of diet or behavior are not represented. However, non-representativeness is less relevant for the present analysis and will likely result in underestimation rather than overestimation of the true associations. On the other hand, the homogeneity of our sample might have reduced our vulnerability to residual confounding. Finally, we examined the long-term relevance of the dietary insulin demand, GI and GL on body composition at a single point in young adulthood only, as presently only 141 participants had at least two anthropometric measurements in both adolescence and young adulthood. In the future, continued follow-up of our participants will also allow analyses of growth trajectories.

A clear strength of our study is its prospective nature and the carefully collected, repeated data on growth, the availability of data on several possible confounders, such as parental character-istics and repeated dietary data. Overall the analyses were based on 1379 weighed 3-day dietary records, that is, on average each subject had provided 5 dietary records during puberty (2–7 records). A further advantage lies in the use of 3-day weighed dietary records, which permitted a particularly detailed assign-ment of dietary GI values for each carbohydrate-containing and FII values for all foods.

In conclusion, our analysis indicates that postprandial increases in insulinemia rather than glycemia are implicated in an unfavorable development of body fat in the critical period of puberty.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

GJ conducted the statistical analysis and wrote the manuscript; GJ and AEB conceived the research project; GJ and JG assigned all FII values to the 3-day weighed dietary records; all authors made substantial contributions to the interpretation of the results.

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Original article

Dietary glycemic load, insulin load, and weight loss in obese, insulin resistant adolescents: RESIST study ☆

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ARTICLE INFO

SUMMARY

Background & aims: The optimal dietary approach for weight loss and improving insulin sensitivity in adolescents is unknown. This study aimed to explore the association between the estimated insulin demand of the diet, as measured by glycemic and insulin load, weight loss, percentage body fat and insulin sensitivity index (ISI) in obese adolescents with clinical features of insulin resistance and/or prediabetes after a 3 month lifestyle and metformin intervention.
Methods: Secondary data analysis of 91 adolescents (median age 12.7 years (range 10.1–17.4) participating in a randomized controlled trial, known as RESIST; ACTRN12608000416392. Weight change between baseline and 3 months was measured by BMI expressed as percentage of the 95th centile (BMI %95). Body composition was measured by dual energy X-ray absorptiometry and ISI was determined by an oral glucose tolerance test.
Results: Higher dietary glycemic load and insulin load were associated with less weight loss (BMI %95), adjusted for sex and pubertal stage, $\beta = 0.0466$, $P = 0.007$ and $\beta = 0.0124$, $P = 0.040$, respectively. Inclusion of total energy intake in the model explained observed associations between dietary glycemic load and insulin load and change in BMI %95. Neither dietary glycemic load nor insulin load were associated with changes in percentage body fat or ISI. Dietary glycemic index and macronutrient content (% of total energy) were not associated to changes in BMI %95, percentage body fat or ISI.
Conclusion: Reduced energy diet contributes to weight loss in obese, insulin resistant adolescents. Diets with a lower insulin demand were associated with a lower energy intake and may hence assist with weight loss.

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1. Introduction

Adolescent obesity is a global public health concern and is associated with a range of health related problems including pre- and type 2 diabetes. Lifestyle interventions, including diet and exercise, with metformin can lead to improvements in weight and insulin sensitivity in adolescents.[1] Yet, little is known about the optimal dietary approach for weight loss in obese adolescents, including those at risk of developing type 2 diabetes.

The conventional therapeutic approach focuses on restricting energy by reducing fat and increasing carbohydrates which may not be the preferred option to treat obese adolescents with insulin resistance. It is speculated that this diet might induce higher levels of postprandial glycemia and/or insulinemia and increase insulin resistance potentially leading to type 2 diabetes.[2,3] Intervention studies in overweight and obese adults indicate an efficacy of low glycemic index and/or glycemic load diets on weight loss[4] especially for individuals with a compensatory increased insulin secretion.[5,6] But there is a paucity of data relating dietary glycemic index or glycemic load and weight loss in obese adolescents and

results from intervention studies are conflicting. One 6 month intervention trial indicated a beneficial effect of dietary glycemic load on weight loss,[7] however, after 3 and 24 month interventions Kirk et al. and Mirza et al., respectively, found that *ad-libitum* diets with reduced glycemic load were as effective as portion size controlled or low fat diets.[8,9]

Dietary glycemic load is considered to be an indirect estimate of insulin demand resulting from carbohydrate containing foods.[10] Nevertheless, insulin secretion is also stimulated by dietary protein. Dietary protein and fat may both act synergistically with carbohydrates to increase insulin levels and reduce glycemia.[11] The new concept of the food insulin index was developed to quantify postprandial insulin responses to all foods including foods with low or no carbohydrate amounts.[10] The average insulin load of the diet can be calculated by summing the product of food insulin index, energy content and consumption frequency over all recorded food items, hence estimating the insulin demand of the overall diet. Novel results from the healthy free living DONALD population suggest that a habitually higher insulin demand during puberty may predispose to higher percentage body fat in adulthood.[12] However, it is unknown whether glycemic or insulin load affects weight loss in obese adolescents with clinical features of insulin resistance.

The aim of this study was to explore the association between the estimated insulin demand of the diet, as measured by glycemic and insulin load and weight loss, percentage body fat and insulin sensitivity index (ISI) in obese adolescents with clinical features of insulin resistance and/or prediabetes after a 3 month lifestyle and metformin intervention.

2. Materials and methods

2.1. Participants

This study is secondary data analysis of a randomized control trial, known as RESIST (Australian New Zealand Clinical Trial Registration Number 12608000416392). The primary aim of RESIST was to determine the efficacy and effectiveness of two structured lifestyle interventions differing in diet composition on insulin sensitivity, in adolescents with clinical features of insulin resistance and/or prediabetes treated with metformin. Prediabetes was defined according to the American Diabetes Association, as impaired fasting glucose 5.6—6.9 mmol/L and/or impaired glucose tolerance 2 h post load 7.8—11.0 mmol/L.[13] Clinical features of insulin resistance were defined as a fasting insulin (pmol/L) to glucose (mmol/L) ratio greater than 20 with one or more of the following: acanthosis nigricans, polycystic ovarian syndrome, hypertension, fasting high-density lipoprotein cholesterol less than 1.03 mmol/L or fasting triglycerides 1.7 mmol/L or greater, as previously described.[14] The design and methods of the study have been previously published[14] as well as the 6 months intention-to-treat analysis.[15] This secondary data analysis was conducted after the adolescents had completed 3 months of intensive dietary intervention.

At baseline 111 participants aged 10—17 years were recruited and randomized to either a high carbohydrate, low fat diet (55%—60% of total energy as carbohydrate (moderate glycemic load), 30% fat, and 15% protein) or a moderate carbohydrate, increased protein diet (40%—45% of total energy as carbohydrate (moderate glycemic load), 30% fat, and 25%—30% protein). Both groups were educated and instructed to consume low-moderate glycemic index foods. Both

diets were prescriptive and two different energy levels were prescribed depending upon age: 6000—7000 kJ (10—14 year olds) or 7000—8000 kJ (15—17 year olds). All participants were commenced on metformin and received the same overall lifestyle intervention. The only difference between the two groups was the macronutrient content of the diets. This study focuses on those 91 participants who completed the initial 3 months of the trial, had at least one assessment of dietary intake and had anthropometry, body composition and insulin sensitivity measured at baseline and 3 months. There was no significant difference in baseline age, anthropometry, body composition or insulin sensitivity between those RESIST participants who included or excluded ($n = 20$) from this study, data not shown. However, there was a higher proportion of females who were not followed up or excluded from this study compared to those participants who were included (85% vs. 54%; $P = 0.010$). The study was approved by The Children's Hospital at Westmead Human Research Ethics Committee (07/CHW/12), Sydney South West Area Health, Western Zone (08/LPOOL/195) and Sydney South West Area Health Service, Royal Prince Alfred Hospital (08/RPAH/455). Written informed consent from parents and assent from the young people was sought prior to their enrolment in the study.

2.2. Anthropometry

Weight and height were measured according to standard procedures. Weight was measured to the nearest 100 g using electronic scales. Height was measured twice, to the nearest 0.1 cm using a wall mounted stadiometer, and the average value was used for data analysis. Body mass index (BMI, kg/m^2) was calculated. Z-scores for weight, height, and BMI were calculated from age and sex specific reference values.[16] BMI was expressed as a percentage of the 95th centile (BMI %95 centile).[17] Overweight and obesity were defined according to the International Obesity Task Force criteria.[18]

2.3. Body composition

Dual energy X-ray absorptiometry (DEXA; Prodigy, Lunar-GE, Madison, WI USA) equipped with propriety software version 13.6 was used to measure body composition. The manufacturer recommended scan mode was used for total body mass measurements. When possible, standard positioning techniques were used. When the participant width exceeded the maximum scan width, they were "mummy wrapped", with arms placed in a lateral position. Scans were analyzed using manufacturer recommended techniques. Repeated measurements in children are often considered unethical, but precision of repeated measurement in adults expressed as the percent coefficient of variation has been shown to be 2.2% for percentage body fat.[19] Fat free mass index (kg of fat free mass/m^2) was calculated.

2.4. Insulin sensitivity

Insulin sensitivity was measured by the ISI determined from an oral glucose tolerance test performed after an overnight fast. The dose of glucose was 1.75 g/kg of body weight to a maximum of 75 g. Plasma glucose and insulin was sampled every 30 min for 2 h as previously described.[14] The ISI was calculated using the following formula[20]:

$$10\,000/\sqrt{\left(\text{insulin}_{fasting} \times \text{glucose}_{fasting}\right) \times (\text{mean 2h glucose} \times \text{mean 2h insulin})}.$$

2.5. Nutritional assessment

Dietary intake was assessed by 24 h dietary recalls using a standardized three-pass methodology[14] which has been previously used in Australian adolescents (2007 Australian National Children's Nutrition and Physical Activity Survey).[21] To assist with estimating the amounts of foods a food model booklet was used.[21] Recalls were collected by trained dieticians, face-to-face in the hospital at weeks 6 and 12 and a telephone interview at week 9. Overall, 213 24 h dietary recalls were included in the analyses and on average participants provided two 24 h dietary recalls (1–3 per participant). The foods consumed were entered into FoodWorks version 6.0.2539 (Xyris Software Inc., Brisbane, QLD 4101, Australia) by research dieticians for nutritional analysis. Macronutrient and energy intake were calculated as means of the 24 h dietary recalls of each participant using the Australian Food and Nutrient Database (AusNut) compiled in 2007 by Food Standards Australia and New Zealand and amended by product and brand specific information using AusNut (AllFoods) and AusNut (Brands) compiled in 1999 by Food Standards Australia and New Zealand.

2.6. Dietary glycemic index and insulin index

Dietary glycemic index is defined as the incremental area under the curve (AUC) of glucose response following the intake of 50 g of carbohydrate from a test food compared with AUC induced by the same amount of carbohydrate ingested as glucose.[22] All carbohydrate-containing foods recorded in the 24 h dietary recalls were assigned a glycemic index value[23] according to a standardized procedure.[24] The content of available carbohydrate (in grams) of each food item was multiplied by the food's glycemic index (as %) to obtain the glycemic load. The sum of glycemic load values for each participant corresponds to the total daily glycemic load. The overall glycemic index of the diet was obtained by dividing the total daily glycemic load by the total daily available carbohydrate intake.

The insulin index of foods is defined as the insulinaemic response (AUC) following the intake of 1000 kJ of a food relative to the insulinaemic response to glucose as reference food. For the present analysis, 121 published insulin index values of foods[10] and 6 recently measured values were available for the assignment to each food recorded according to a standardized procedure.[12] Foods which could not be assigned an insulin index value of a food following this procedure (3% of the foods) were assigned a median insulin index value of its corresponding food group based on the 2007 Australian National Children's Nutrition and Physical Activity Survey. The participant's average dietary insulin load was calculated by summing the product of insulin index values of foods, energy content and consumption frequency over all recorded food items in the 24 h dietary recall.

2.7. Metformin

All participants received metformin therapy. The initial dose was 250 mg twice a day. After the first two weeks this was increased to a final dose of 500 mg daily. Metformin compliance was assessed by pill counts by the clinical trials pharmacist at 3 months. From the participants of this study sample 60 participants (66%) returned pills. From the pill count, it was estimated that participants who lost weight consumed [median (interquartile range, IQR)] 88% (66, 99) of the prescribed metformin, and participants who did not lose weight 90% (56, 96). Metformin compliance did not differ between participants who lost weight and those who did not ($P = 0.782$).

2.8. Statistical analysis

All statistical analyses were carried out using SAS (version 9.2, SAS Institute, Cary, NC, USA). A P-value <0.050 was considered as statistically significant.

Change in weight status was measured by change in BMI expressed as BMI %95 centile. Participants were categorized according to their weight loss from baseline to 3 months, i.e. participants who lost weight (BMI %95 centile decreased) and participants who did not lose weight (BMI %95 centile increase ≥0%). Differences between groups were analyzed using independent sample *t*-test for normally distributed continuous variables and Wilcoxon rank sum test for non-normally distributed continuous variables, chi-square-test for categorical variables, and Fisher Exact test for categorical variables if ≤50% of cells had expected counts less than 5.

A linear regression analysis was conducted (using the general linear models procedure) to adjust dietary glycemic load and insulin load of participants who lost weight and those who did not for total energy intake. In addition, we ran linear regression analysis, pooling data from all participants, to analyze whether changes in dietary glycemic load or insulin load, as well as dietary glycemic index and macronutrient content (% of total energy) during the dietary intervention (average of values used from weeks 6, 9, and 12) were associated with changes in BMI %95 centile, percentage body fat, and ISI from baseline to 3 months. Furthermore, we ran two sets of conditional models. First, we analyzed the relation between dietary glycemic load or insulin load and BMI %95 centile and then introduced total energy intake as a pathway variable. Second, we analyzed the relation between total energy intake and BMI %95 centile and then introduced dietary glycemic load or insulin load as a pathway variable. All models conform to the assumptions of linear regression models (linearity, normality and homoscedasticity of residuals, absence of multicollinearity).

3. Results

Baseline general characteristics and nutritional characteristics during the intervention of the participants are shown in Table 1. Overall the median change in BMI %95 centile over the 3 month intervention was −6.5 (IQR: −9.7, −2.5), 79 participants decreased BMI %95 centile and 12 participants increased BMI %95 centile. The total median change in percentage body fat and ISI was −1.3 (−3.1, 0.01) and 0.29 (−0.08, 0.67), respectively. Participants who lost weight were significantly younger (2.2 years) and tended to be taller than participants who did not lose weight. There were no other statistically significant differences in general characteristics between participants who did lost weight and those who did not (Table 1).

The median energy intake of all participants was 6.3 MJ/d, carbohydrate, protein and fat contributing 47.7%, 20.1%, and 29.4% of total energy, respectively. No differences in macronutrient intake (% of total energy) were observed between the participants who lost weight and those who did not. However, participants who lost weight reported consuming 1.4 MJ less energy (Table 1) which corresponded to a significantly ($P < 0.001$) lower absolute intake of carbohydrate (mean difference 26.1 g), protein (27.0 g) and fat (29.4 g) compared to participants who did not lose weight. The dietary glycemic index did not differ between participants who lost weight and those who did not. But on average, participants who lost weight had a significantly lower dietary glycemic load and insulin load of 18 and 37 points, respectively compared to participants who did not lose weight ($P = 0.013$ and $P = 0.003$, respectively, Table 1). After adjusting for energy intake, the dietary glycemic load and insulin load were no longer different between

Table 1
Baseline general characteristics and nutritional characteristics during the intervention of participants stratified by change in weight status expressed as BMI %95 centile after 3 months of intervention. Categories are participants who did not lose weight (BMI %95 centile increase ≥0%) and participants who lost weight (BMI %95 centile decreased).

	Non-weight loser	Weight losers	P value[a]
n	12	79	
Median change in BMI %95 centile (%)	1.3 (0.8, 1.8)	−7.7 (−10.1, −1.4)	<0.001
General characteristics			
Age (years)	14.9 (11.9, 15.9)	12.7 (11.6, 14.1)	0.021
Female, n (%)	4 (33.3)	45 (57.0)	0.126
Pubertal stage, n (%)[b]	7 (58.3)	52 (66.7)	0.745
Anthropometry			
Weight z-score	2.49 ± 0.76	2.70 ± 0.53	0.240
Height z-score	0.63 ± 1.59	1.36 ± 1.09	0.046
BMI z-score	2.30 ± 0.32	2.34 ± 0.29	0.598
BMI %95 centile	125 (118, 139)	127 (117, 143)	0.721
Obese, n (%)[c]	11 (91.7)	76 (96.2)	0.438
Body composition (DEXA)			
Total body fat (%)	48.8 ± 5.5	48.5 ± 5.5	0.862
Fat free mass (kg)	45.3 (34.7, 55.8)	41.6 (35.7, 52.6)	0.602
Fat free mass index (kg/m^2)	15.9 (14.4, 18.7)	17.5 (14.6, 18.4)	0.703
Clinical and metabolic profile			
Fasting blood glucose (mmol/L)	5.0 (4.6, 5.4)	4.7 (4.4, 5.0)	0.064
Fasting insulin (pmol/L)	225 (186.5, 250.5)	244 (173.1, 311.0)	0.483
Insulin sensitivity index[d]	1.35 (1.01, 1.62)	1.23 (0.84, 1.57)	0.635
Prediabetic, n (%)[e]	1 (8.3)	8 (10.1)	1
Nutritional characteristics			
Total Energy (MJ/d)	7.5 (6.8, 9.0)	6.1 (5.3, 6.8)	<0.001
Fat (% of energy)	32.0 (6.8)	29.7 (6.4)	0.255
Protein (% of energy)	20.7 (4.8)	20.3 (4.2)	0.752
Carbohydrate (% of energy)	45.8 (6.6)	48.2 (6.8)	0.247
Dietary glycemic index	54.5 (4.8)	54.4 (4.1)	0.933
Dietary glycemic load	111 (104, 122)	93 (78, 111)	0.013
Dietary insulin load	324 (302, 365)	287 (242, 312)	0.003

Values are mean ± standard deviation, median (interquartile range).
BMI, body mass index; BMI %95 centile, percentage BMI of the 95th centile.
[a] Significant differences between participants who lost weight and those who did not were tested using t-test for normally distributed continuous variables, Wilcoxon rank sum test for not normally distributed continuous variables, Chi-square test for categorical variables, and Fisher Exact test if ≤50% of cells have frequencies under 5.
[b] Tanner stage ≥3.
[c] Derived from the age- and sex-specific cut-points proposed by the International Obesity Task Force,[18] which are linked to the adult cut-off point of a BMI of 25 kg/m^2.
[d] Derived from Matsuda and DeFronzo.[20]
[e] Derived from the American Diabetes Association.[13]

groups (glycemic load: 95 vs. 96, P = 0.917; insulin load: 282 vs. 282, P = 0.987).

The reported median dietary glycemic index and glycemic load of participants who consumed a high carbohydrate, low fat diet did not differ significantly to those reported by participants consuming a moderate carbohydrate, increased protein diet (glycemic index: 53.2 vs. 54.1 and glycemic load: 93.4 vs. 98.0, respectively; P for all >0.050).

Analysing data as continuous variables showed that a higher dietary glycemic load and higher dietary insulin load were associated with less weight loss between baseline and 3 months, even after the adjustment for sex and pubertal stage (glycemic load: P = 0.007, insulin load: P = 0.040; Table 2). In fact, an increase in dietary glycemic load by 50 units was associated with a 2.3% increase in BMI %95 centile, while an increase in dietary insulin load by 50 units was associated with a 0.6% increase in BMI %95 centile. Further adjustment for ethnicity, parental education, baseline ISI or change in ISI did not significantly change the results. Including total energy intake within an additional mediation model explained the associations between dietary glycemic load or insulin load and weight loss between baseline and 3 months (glycemic load:

Table 2
Associations between dietary glycemic load, insulin load and weight change expressed as a percentage of the 95th centile (BMI %95 centile, in %) of the RESIST study (n = 91).

Predictors	β	SE	R^2	P value
Glycemic load				
Unadjusted	0.0436	0.0167	0.07	0.011
Model A	0.0466	0.0170	0.11	0.007
Model B	0.0219	0.0269	0.13	0.419
Insulin load				
Unadjusted	0.0128	0.0059	0.05	0.032
Model A	0.0124	0.0059	0.08	0.040
Model B	−0.0137	0.0132	0.13	0.302

Model A: Adjustment for sex and pubertal status (n = 90, one missing for pubertal stage).
Model B: Model A plus adjustment for total energy intake.
n = 90 for Model A and B: one missing for pubertal status at baseline.

P = 0.419, insulin load: P = 0.302; Table 2). Dietary glycemic load and insulin load were not associated with changes in percentage body fat or ISI between baseline and 3 months and the adjustment of percentage body fat for insulin sensitivity vice versa did not change the results, data not shown.

Moreover, dietary glycemic index and macronutrient content (% of total energy) of the diet were not associated to changes in BMI % 95 centile, percentage body fat or ISI between baseline and 3 months and the adjustment of percentage body fat for insulin sensitivity and vice versa did not change the results, data not shown.

3.1. Sensitivity analyses

We performed additional sensitivity analyses using only data from the 78 participants (85.7%) who had provided two or more 24 h dietary recalls during the intervention and all results were similar, data not shown. Using only data from 24 h dietary recalls collected at weeks 6 and 9 (n = 79; 86.8%) yielded comparable results for dietary glycemic load. However, dietary insulin load was significantly associated with weight loss in the unadjusted model only (P = 0.046). Further adjustments for sex and pubertal stage attenuated the relation (P = 0.070).

3.2. Conditional models

Introducing total energy intake in the model attenuated the association between glycemic load or insulin load and change in BMI %95 centile between baseline and 3 months (as described, Table 2). Hence, total energy intake may lie on the pathway between dietary glycemic load or insulin load and change in BMI %95 centile. Introducing dietary glycemic load attenuated the association between total energy intake change in BMI %95 centile between baseline and 3 months (P = 0.005 and P = 0.419, respectively), yet introducing dietary insulin load did not notably attenuate the association between total energy intake and change in BMI %95 centile between baseline and 3 months (from P = 0.005 to P = 0.030). Therefore, the results do not consistently suggest a mediation of dietary energy intake and change in BMI %95 centile between baseline and 3 months by dietary glycemic load or insulin load.

4. Discussion

To our knowledge this is the first study to examine the association between dietary insulin demand, measured by glycemic load and insulin load, and weight loss in obese adolescents with clinical features of insulin resistance and/or pre-diabetes. The central

finding of our secondary data analysis was that participants who lost weight over 3 months reported a lower energy diet, with a reduced insulin demand compared to participants who gained weight. There was no significant difference in the glycemic index or macronutrient content (% of total energy) of the diet between those who those who lost weight and those who did not.

Our findings relating to glycemic load and weight loss are broadly consistent with previous, but limited, trials indicating that low glycemic load diets, compared to reduce fat diets are effective in promoting weight loss in obese children and adolescents.[7,25] Not all studies support this association; no differences were observed in weight loss among children when a low glycemic load diet was compared to a portion controlled or low fat diet after 3 and 24 month interventions.[8,9] Mechanisms linking glycemic load to weight loss are based on postprandial insulin response, which is though captured by glycemic load,[10] may be better described by insulin load itself. Foods and/or diets producing lower postprandial insulin responses are considered to induce higher satiety and a lower voluntary food intake at a subsequent meal, compared to foods inducing a high insulin demand.[26] We speculate that RESIST participants consuming a lower glycemic load or insulin load diet may have increased satiety after eating, which in turn facilitated a reduction in energy intake.[7]

After adjustment for total energy intake, glycemic load and insulin load did not differ between participants who lost weight and those who did not. It is thus possible that the unadjusted association between glycemic load or insulin load and BMI %95 simply reflects the fact that lower energy intake was accompanied by a lower glycemic load and insulin load. However, the conditional models support a mediation of the association between glycemic load or insulin load and BMI %95 by energy, i.e. that lowering glycemic load or insulin load facilitated a reduction of overall total energy intake.

In contrast to a recent systematic review, which concluded that consuming a low glycemic index diet, not a low glycemic load diet, had favorable effects on reducing energy intake and subsequent obesity in children and adolescents,[27] we did not observe any relation between dietary glycemic index and weight loss. Dietary glycemic index is a qualitative rather than quantitative measure indicating the ranking of postprandial glucose and insulin responses to foods.[22] We postulate that reducing the insulin demand of the diet might be more important in our study population with compromised glycemic status, as they need to adapt their insulin secretion with increased amounts of carbohydrates, compared to metabolically healthy obese children. In addition, it could be argued that the variation of the dietary glycemic index was too small to detect any relation, because all participants were instructed to follow a moderate glycemic index diet.

We also found no significant association between glycemic load, insulin load or glycemic index and percentage body fat. A recent cohort study including healthy participants found that a higher dietary insulin demand during puberty, estimated by dietary insulin load, was prospectively associated with higher percentage body fat in young adulthood.[12] Postprandial insulin levels may direct nutrients from oxidation in the muscle towards storage in fat as well as restrain hepatic glucose production and suppress lipolysis. We speculate that the lack of a relation with regards to body fat may stem from the short study time and/or hormonal effects of pubertal participants, particularly in girls who may be expected to increase their fat mass during the pubertal growth spurt.

Associations between dietary glycemic load and glycemic index and measures of insulin sensitivity have been inconsistently reported. Two 6 month intervention studies among obese children and adolescents observed significant decreases in insulin resistance with a reduced glycemic load diet without energy restriction[7] and within

an energy restricted low glycemic index group only.[28] By contrast, a 12 months intervention among obese Hispanic children did not show any association between dietary glycemic load and insulin sensitivity.[9] Similarly, we did not observe any relation between dietary glycemic load, insulin load or glycemic index and ISI.

This study had a number of limitations. Firstly, it was not clearly prospective as the average dietary intake was calculated from 24 h dietary recalls at weeks 6, 9, and 12 to examine associations with outcomes at 3 months and not all participants completed 3 recalls. It is not possible to draw a conclusion regarding cause and effect because this study is purely observational. Secondly, dietary intake was self-reported diet assessment and hence may not accurately represent food intake. Furthermore, the methodology of three-pass 24 h dietary recalls is not as precise as weighed dietary records, but suitable for this young study population and has been used in the 2007 Australian National Children's Nutrition and Physical Activity Survey.[21] Thirdly, we applied the food insulin index concept, developed to quantify the insulin response to foods – to estimate the dietary insulin demand. Hence, the limitations debated for the estimations of dietary glycemic index also apply to the estimation of dietary insulin demand. The assignment of insulin index values of foods was based on 127 values only and therefore it must be considered crude. Finally, the specific effect of metformin therapy on outcome measures is not clear. Evidence indicates that metformin therapy combined with lifestyle interventions can lead to improvements in weight status and insulin sensitivity in adolescents with clinical features of insulin resistance,[1] even though the effects of metformin therapy alone are conflicting.[29,30] Since all RESIST participants received the same metformin dose, a potential influence of metformin would be expected to be comparable across all participants.

A strength of this study is the recruitment and high retention rate of a high risk study population – obese adolescents with features of insulin resistance and/or prediabetes. Dietary compliance was assessed using three scheduled 24 h dietary recalls at weeks 6, 9, and 12 and the estimated energy intake from the recalls was broadly consistent with the prescribed diet[14] i.e. the median (interquartile range)reported energy intakes during the 3 months for the were 6.1 MJ (5.3, 7.2) for the 10–14 year old participants and 6.6 MJ (6.2, 6.9) for the 15–17 year old participants.

To date, this is the first and largest study to examine dietary insulin demand in a high risk population. Moreover, the study was well controlled and participants were supported by dieticians to follow prescribed diets.

In conclusion, our data supports the increasing body of evidence that a reduced energy diet contributes to weight loss in obese adolescents at high risk of type 2 diabetes. Lower dietary glycemic load and insulin load diets were associated with a lower energy intake and may hence assist with weight loss.

Grants and fellowships

Statement of authorship

The contributions of the authors were as follows: GJ: conducted the statistical analysis and wrote the manuscript; GJ, SPG, and AEB:

conceived the research project; GJ and JG assigned all insulin index values of foods to the 24 h dietary recalls; all authors: made substantial contributions the interpretation of the results.

Conflict of interest

The authors declare no conflict of interest.

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135

Prospective Association of Protein Intake During Puberty with Body Composition in Young Adulthood

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Objective: To examine the association of habitual animal and plant protein intake during the potentially critical period of puberty with body composition in young adulthood.

Design and Methods: Multivariable regression analyses were performed on data from 140 female and 122 male participants of the DONALD Study with ≥ 2 3-day weighed dietary records during puberty (girls 9-14 years; boys 10-15 years) and anthropometric measurements in young adulthood (18-25 years). Fat-free mass index (FFMI) and fat mass index (FMI) were estimated from four skinfolds.

Results: In women, a higher pubertal animal protein consumption was independently related to higher levels of FFMI ($p_{trend} = 0.001$), but not to FMI ($p_{trend} = 0.5$). Adjusted means of FFMI in energy-adjusted tertiles of animal protein intake were 15.3 (95% confidence interval: 15.0, 15.5), 15.4 (15.1, 15.7), 16.2 (15.9, 16.6) kg/m². In men, a higher animal protein intake was related to a higher FFMI ($p_{trend} = 0.04$) and a lower FMI ($p_{trend} = 0.001$) only after adjusting FFMI for current FMI levels and vice versa. Plant protein was not associated with body composition among either sex.

Conclusions: Our results show that a higher pubertal animal protein consumption may yield a higher fat-free mass in young adulthood.

Introduction

Substantial controversy exists concerning a potential effect of dietary protein intake on body mass and body composition. A beneficial effect of a higher dietary protein content has been observed in a number of weight loss- and weight control-trials (1-5). On the other hand, some evidence from prospective observational studies points to a detrimental effect of (animal) protein intake on body weight and body mass index (BMI) in adults over the long term (6-9). While it is plausible that these two study types—with their different designs, contexts, and times of duration—yield diverging results, the issue of how long-term protein intake relates to health remains far from being solved.

The particularities of study designs are not the only obstacle in assessing the evidence. The limited validity of body weight or BMI as proxies for body fat may be of special relevance with regard to dietary protein: In a recent randomized controlled trial (RCT) with healthy young adults (10), overeating on a low protein diet produced less weight gain than overeating on a diet with a normal or high protein content. Yet, the additional weight gained on the higher protein diets stemmed from fat-free mass only. Subsequently, a link of higher protein intakes to higher body weight may not be specific to fat mass. Puberty is a developmental phase during which major changes in body composition occur. It is possible that, because of the anabolic nature of metabolism during this phase (11), a diet that is relatively high in protein favors a body composition characterized by a higher fat-free mass.

In this study, we investigated the association of habitual protein intake during puberty with fat mass index (FMI) and fat-free mass index (FFMI) in young adulthood. A secondary aim was to consider protein intake during early childhood (age 12-24 months) and adiposity rebound (age 4-6 years) as these windows represent, similar to puberty, potentially critical periods for later obesity risk (12,13).

Methods and Procedures

Study population

The present study was ancillary to the **Do**rtmund **N**utritional and **A**nthropometric **L**ongitudinally **D**esigned **S**tudy (DONALD Study), an ongoing, open cohort study conducted in Dortmund, Germany. Details on this study have been described elsewhere (14). Briefly,

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since the recruitment began in 1985, detailed data on diet, growth, development, and metabolism between infancy and adulthood have been collected from over 1300 healthy children. The study was approved by the Ethics Committee of the University of Bonn, and all examinations are performed with parental consent.

Because of the open cohort design, many DONALD participants have not yet reached young adulthood. In total, data from 394 subjects aged > 18 years were available for this analysis. These subjects were term (37-42 week gestation) singletons with a birth weight > 2500 g and had at least one anthropometric measurement taken in young adulthood ($\geq$ 18 and $\leq$ 25 years of age, mean age = 20.3 years), of which we used the last for the present analysis. Three hundred and eight of these participants had provided at least two 3-day weighed dietary records during puberty (girls 9-14 years, boys 10-15 years). Participants who consistently underreported their energy intake (i.e. who had provided more implausible (15,16) than plausible food records) were excluded from the study (*n* = 23). Furthermore, participants had to have anthropometric data at puberty and information on relevant covariates such as early life and socioeconomic factors. This resulted in a final sample of 262 participants (53.6% female, 46.4% male). Overall, 1376 food records were included in the present analysis (2-7 records per participant, on average five per subject). Concerning our additional analyses on the associations of protein intake during early childhood and adiposity rebound with adult body composition, data were available for 159 participants (86 males, 73 females) and 220 participants (107 males, 113 females), respectively. As these sample sizes are quite small and as our main focus was puberty, the respective results are not presented in detail.

Anthropometric measurements

Participants are measured at each visit according to standard procedures (17), dressed in underwear only and barefoot. From the age of 2 years onward, standing height is measured to the nearest 0.1 cm using a digital stadiometer (Harpenden, Crymych, UK). Body weight is measured to the nearest 100 g using an electronic scale (Seca 753E; Seca Weighing and Measuring Systems, Hamburg, Germany). Skinfold thicknesses are measured from the age of 6 months onward at four different sites (suprailiacal, subscapular, biceps, triceps) on the right side of the body to the nearest 0.1 mm using a Holtain caliper (Holtain, Crosswell, United Kingdom). The three trained nurses performing the measurements undergo annual quality controls, conducted in six to eight healthy young adult volunteers. Average inter- and intra-individual variation coefficients obtained in the last seven years (2005-2011) were 9.1% and 12.8% for biceps, 5.0% and 5.9% for triceps, 5.1% and 7.6% for subscapular, and 8.4% and 8.5% for supra-iliacal skinfolds.

Anthropometric calculations

Body fat mass and fat-free body mass were calculated as "(percentage body fat (%BF) * body mass) /100" and "((100 − %BF) * body mass) /100", respectively, and related to height to obtain the indices FMI and FFMI (kg/m^2). %BF at puberty was derived using equations of Slaughter et al. for pubescent children (18), which consider triceps and subscapular skinfolds. %BF in young adulthood was estimated from skinfolds using Durnin and Womersley equations, which are based on triceps, biceps, scapular and iliacal skinfolds (19). We chose to investigate FMI and FFMI rather than %BF as the use of

this measure has recently been criticized to incorrectly reflect body-size-adjusted adiposity (20).

Nutritional assessment

Food consumption in the DONALD Study is assessed annually using 3-day weighed dietary records. All foods and beverages consumed are weighed and recorded, as well as leftovers, to the nearest 1 g over three days using electronic food scales (initially Soehnle Digita 8000; Leifheit SG, Nassau; Germany; now WEDO digi 2000; Werner Dorsch GmbH, Muenster/Dieburg, Germany). For this analysis, dietary variables were calculated as individual means of the 3-day weighed dietary records using LEBTAB, the in-house database, which is continuously updated to include all recorded food items. LEBTAB is based on the German standard food tables (21) and data obtained from commercial food products. Individual average intakes were calculated from at least two records during puberty. In a validation study conducted with data from DONALD participants, the correlation coefficient between protein intake as determined by 3-day weighed records and as determined by 24-h-urinary excretion was 0.59 among 11-13 year olds (22). In order to create the food groups "dairy protein" and "meat protein", foods were broken down into their components as appropriate (e.g. a pizza was broken down into dairy products, meat products, cereal products, and other product groups).

Early life and socioeconomic characteristics

On their child's admission to the study, parents are interviewed by the study paediatrician, and weighed and measured by the study nurses using the same equipment as for children from two years onward. Information on the child's birth characteristics is abstracted from the 'Mutterpass', a standardized document given to all pregnant women in Germany. The duration of full breastfeeding (no solid foods or liquids other than breast milk, tea or water) is inquired by dieticians at the first visits. For this analysis, the following early life and socioeconomic characteristics were considered as potentially confounding factors: breastfeeding status (fully breastfed (yes/no), defined as fully breastfed > two weeks), birth weight (two variables were tested: < 3000 g vs. $\geq$ 3000 g and < 3500 g vs. $\geq$ 3500 g (an approximate median split)) maternal overweight status (BMI $\geq$ 25 kg/m^2) and high maternal educational status ($\geq$ 12 years of schooling).

Statistical analysis

Participant characteristics are presented by gender and energy-adjusted tertiles of animal protein intake at puberty. Tests for differences across tertiles were performed using Kruskal-Wallis tests for continuous variables and χ^2-tests for categorical variables. The association between diet during puberty and body composition in young adulthood was analyzed by multiple linear regression models. As FMI and FFMI in puberty and young adulthood were not normally distributed, they were log-transformed (FMI) or 1/x-transformed (FFMI). To account for the major gender-specific differences in the development of body composition (11), all analyses were stratified by sex. Age at the measurement in young adulthood and the respective baseline anthropometric variable (FMI, FFMI) were included in basic models (Model A). Early life and socioeconomic factors were considered separately and included if they modified the respective association substantially (that is, if their inclusion caused a change

in the regression coefficient for protein intake of $> 10\%$ (23)). In a further step, we additionally adjusted for nutritional factors using the same criterion (model B). Here, we merely considered total energy intake and nutritional factors which do not provide energy (dietary glycemic index, dietary fiber, calcium), so as to avoid presenting estimates that partially reflect the substitution of protein for other macronutrients. Instead, we ran additional models that explicitly assess the effect of a substitution of animal/plant protein for carbohydrates or for fats. To simulate substitution effects, total energy and the energy-bearing nutrients to be held constant (fats and plant/animal protein or carbohydrates and plant/animal protein, respectively) were included in the models (24). We only present substitution models for associations identified as significant in fully adjusted analyses (model B).

In order to understand more specifically how protein intake was related to fat-free mass—independently of current fat mass—and vice versa, we ran additional analyses in which we adjusted adult FFMI levels for adult FMI levels and adult FMI levels for adult FFMI levels.

All dietary variables were energy-adjusted using the residual method (24). To account for age-dependent changes in intake levels, all variables were standardized by age group and sex. In addition to multiple linear regression analyses, which were used to obtain information on linear trends ($p_{for\ trend}$), we conducted analyses of covariance. Here, protein intake was included as a categorical variable (in the form of energy-adjusted tertiles) to obtain adjusted means (least-squares means) of FMI and FFMI in young adulthood by tertiles of protein intake. A P-value < 0.05 was considered statistically significant. All analyses were carried out using SAS procedures (version 9.1.3, SAS Institute, Cary, NC, USA).

Results

In **Table** 1, early-life and familial characteristics and anthropometric data of the 140 female and the 122 male participants are presented by energy-adjusted tertiles of animal protein intake during puberty. Male participants in the highest tertile had a higher FFMI at baseline, but did not differ in their adult anthropometry. Females in the highest tertile had higher pubertal levels of BMI as well as higher adult levels of BMI, FMI, and FFMI and were the most likely to be overweight in young adulthood. Moreover, they were least likely to have a mother with a high educational status.

Nutritional data at baseline by tertiles of animal protein intake are presented in **Table** 2. As expected, intakes of protein and calcium differed notably between tertiles in both genders. Similarly, carbohydrate nutrition was associated with animal protein, except for fiber intake levels. Of note, higher animal protein intakes were related to higher total fat and saturated fat intakes in females only.

Pubertal protein intake and adult body composition
Among females, neither intake of animal protein nor intake of plant protein during puberty was related to FMI in young adulthood in our main multivariable analyses (**Table** 3. However, a higher intake of animal protein during puberty was associated with a higher FFMI

in young adulthood (**Table** 4, $p_{for\ trend} = 0.001$). Substitution models revealed that consuming more energy from animal protein while consuming less energy from carbohydrates was significantly related to a higher FFMI among females ($p_{for\ trend} = 0.01$; adjustment for age, baseline FFMI, breastfeeding, maternal education status, calcium, fats, plant protein, and energy; data not shown). Concerning a substitution of animal protein for fats, we observed only a trend ($p_{for\ trend} = 0.08$; adjustment for age, baseline FFMI, breastfeeding, maternal education status, glycemic index, calcium, carbohydrates and plant protein; data not shown). In males, animal or plant protein intake during puberty was neither related to FMI nor to FFMI (**Table** 3. Further adjustment for a variable describing the presence/absence of smokers in the family household (yes/ no) did not change our main results (within the subset of participants for which the variable was available, ($n = 242$)).

To acquire additional information on the relevant protein source driving the association between animal protein intake and FFMI in females, we investigated two major sources of animal protein: meat and dairy foods. A higher intake of protein from meat during puberty was related to a higher FFMI in young adulthood (adjusted means of FFMI within tertiles of meat protein intake: 15.4 (15.1-15.7), 15.5 (15.2-15.8), 16.0 (15.7-16.4) kg/m^2; $p_{for\ trend} = 0.002$). There was no significant relation of dairy protein intake during puberty and FFMI in young adulthood (adjusted means of FFMI within tertiles of milk protein intake: 15.7 (15.4-16.0), 15.6 (15.3-16.0), 15.5 (15.2-15.8) kg/m^2; $p_{for\ trend} = 0.17$).

Pubertal protein intake and FMI at fixed levels of FFMI and vice versa
Additional analyses in which we adjusted FMI for current FFMI and vice versa (**Table** 4 yielded similar results for the association between animal protein and FMI or FFMI among females. In men, on the other hand, a higher consumption of animal protein during puberty was now significantly related to a lower FMI ($p_{for\ trend} = 0.001$) and to a slightly higher FFMI in young adulthood ($p_{for\ trend} = 0.04$). Plant protein remained unrelated to adult body composition among both sexes.

Protein intake during earlier phases of life and body composition in young adulthood
We observed no associations between protein intake during early childhood and body composition in young adulthood (all $p_{for\ trend}$-values ≥ 0.3). By contrast, dietary animal protein during the period of adiposity rebound tended to be related to FFMI in young adulthood among males (adjusted means of FFMI within tertiles of animal protein intake: 18.8 (18.1-19.5), 19.7 (19.0-20.4), 19.7 (19.0-20.5) kg/m^2; $p_{for\ trend} = 0.05$), but not in females [adjusted means: 15.5 (14.9-16.1), 16.1 (15.6-16.6), 16.2 (15.6-16.8) kg/m^2; $p_{for\ trend} = 0.13$]. Animal or plant protein in the period of adiposity rebound was not related to adult FMI.

Discussion

Our data suggest a link between a higher pubertal animal protein consumption and a higher adult FFM, primarily among women. In terms of the driving force of this association, a substitution of animal protein for carbohydrates seemed to be slightly more relevant

TABLE 1 Demographic, anthropometric, birth, and socioeconomic characteristics by energy-adjusted tertiles of animal protein intake during puberty

	Total n	Males (n = 122)				Females (n = 140)			
		Tertile 1 (n = 40)	Tertile 2 (n = 40)	Tertile 3 (n = 41)	P^a	Tertile 1 (n = 46)	Tertile 2 (n = 47)	Tertile 3 (n = 47)	P^a
Young adulthood characteristics[b]									
Age (years)	262	18.2 (18.0, 22.1)	22.0 (18.1, 22.7)	21.2 (18.0, 22.1)	0.2	19.1 (18.3, 22.4)	19.0 (18.1, 21.9)	19.0 (19.0, 22.5)	0.5
Overweight, n (%)[c]	262	13 (32.5)	15 (36.6)	9 (22.0)	0.3	5 (10.9)	2 (4.3)	10 (21.3)	0.04
BMI (kg/m^2)	262	23.1 (20.4, 25.4)	23.4 (20.7, 26.1)	23.2 (21.1, 25.0)	0.8	21.5 (19.4, 22.9)	20.7 (19.7, 23.4)	22.4 (21.4, 24.8)	0.001
FMI (kg/m^2)	262	4.1 (2.7, 5.5)	4.0 (2.9, 5.6)	3.9 (3.0, 4.8)	0.7	6.2 (5.0, 7.3)	6.0 (4.8, 7.2)	6.7 (6.1, 7.8)	0.02
FFMI (kg/m^2)	262	18.7 (17.3, 19.8)	19.4 (18.3, 20.2)	19.2 (18.3, 20.3)	0.18	15.3 (14.2, 16.1)	15.1 (14.4, 16.2)	15.9 (15.3, 17.3)	0.001
Pubertal characteristics[d]									
Age (years)	262	10.0 (10.0, 10.0)	10.0 (10.0, 10.1)	10.0 (10.0, 10.1)	0.4	9.0 (9.0, 9.1)	9.0 (9.0, 9.1)	9.0 (9.0, 9.1)	0.4
BMI (kg/m^2)	262	16.2 (16.3, 18.3)	17.4 (15.9, 18.2)	17.7 (16.2, 18.8)	0.12	16.0 (15.1, 17.1)	16.3 (14.9, 17.4)	17.3 (15.6, 19.0)	0.049
FMI (kg/m^2)	262	2.1 (1.6, 3.7)	2.6 (1.8, 3.4)	2.5 (1.9, 3.5)	0.3	2.7 (2.3, 3.3)	2.7 (2.1, 3.6)	3.0 (2.3, 4.4)	0.3
FFMI (kg/m^2)	262	13.9 (13.4, 14.6)	14.5 (13.5, 15.1)	15.0 (13.9, 15.7)	0.02	13.3 (12.6, 14.0)	13.4 (12.8, 13.8)	13.8 (13.0, 14.6)	0.06
Early life characteristics									
Birth weight ≥ 3500g, n (%)	262	26 (65.0)	23 (56.1)	18 (43.9)	0.16	22 (47.8)	17 (36.2)	17 (36.2)	0.4
Fully breastfed > 2 weeks, n (%)	262	30 (75.0)	25 (61.0)	32 (78.1)	0.19	32 (69.6)	37 (78.7)	29 (61.7)	0.2
Family characteristics									
Maternal overweight, n (%)[c]	262	9 (22.5)	14 (34.2)	14 (34.2)	0.4	15 (32.6)	13 (27.7)	19 (40.4)	0.4
Smoking in the household, n (%)	242	8 (21.6)	18 (48.7)	11 (28.2)	0.04	14 (33.3)	18 (41.9)	13 (29.6)	0.5
Mother ≥ 12 y schooling, n (%)	262	23 (57.5)	10 (24.4)	23 (56.1)	0.003	22 (47.8)	25 (53.2)	12 (25.5)	0.02

DONALD Study, Germany
Abbreviations: BMI, body mass index; FMI, fat mass index; FFMI, fat-free mass index.
Values are medians (25th percentile, 75th percentile) for continuous variables and **n** (%) for categorical variables.
[a]Differences between the tertiles were tested using a Kruskal-Wallis test for continuous variables and χ^2-test for categorical variables. **P**-values ≥ 0.2 with one decimal.
[b]18−25 years; one measurement available per participant.
[c]BMI ≥ 25 kg/m^2.
[d]Girls 9−14 years, boys 10−15 years; we used the first measurement available.
FMI and FFMI were derived on the basis of skinfold thicknesses. Height, weight, and skinfold measurements were conducted by trained nurses.

APPENDIX 3

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EPIDEMIOLOGY/GENETICS

Obesity

140

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TABLE 2 Nutritional data by energy-adjusted tertiles of animal protein intake during puberty

	Total n	Males (n = 122)				Females (n = 140)			
		Tertile 1 (n = 40)	Tertile 2 (n = 40)	Tertile 3 (n = 41)	P^a	Tertile 1 (n = 46)	Tertile 2 (n = 47)	Tertile 3 (n = 47)	P^a
Total energy (kcal)	262	2139 (1838, 2396)	2086 (1945, 2343)	2039 (1920, 2304)	0.9	1738 (1565, 1887)	1703 (1568, 1890)	1711 (1584, 1902)	0.95
Fat (% of energy)	262	35.7 (32.5, 38.0)	36.2 (33.9, 38.4)	35.9 (33.2, 38.1)	0.6	34.7 (32.1, 37.0)	35.4 (33.1, 37.1)	37.6 (35.7, 39.9)	0.0005
Saturated fatty acids (% of energy)	262	15.0 (13.1, 16.7)	15.9 (14.9, 16.8)	15.5 (14.2, 17.0)	<.0001	15.6 (13.9, 16.6)	15.4 (14.2, 16.3)	16.4 (15.3, 17.8)	0.01
Protein (% of energy)	262	12.0 (11.3, 12.6)	13.3 (12.7, 13.7)	14.6 (14.0, 15.4)	<.0001	11.2 (10.9, 11.8)	12.8 (12.2, 13.4)	14.4 (13.4, 15.2)	<.0001
Animal protein (% of energy)	262	7.0 (6.6, 7.5)	8.4 (8.1, 8.8)	10.0 (9.5, 10.6)	<.0001	6.4 (5.9, 6.6)	7.9 (7.7, 8.2)	9.6 (9.0, 10.4)	<.0001
Meat protein (% of energy)	262	2.6 (2.0, 3.3)	3.3 (2.8, 3.9)	4.3 (3.1, 5.5)	<.0001	2.0 (1.3, 2.5)	3.0 (2.3, 4.0)	4.2 (3.0, 4.9)	<.0001
Dairy protein (% of energy)	262	3.4 (2.5, 4.3)	4.2 (3.7, 4.7)	4.8 (3.8, 5.8)	0.0001	3.4 (3.0, 3.9)	3.8 (3.1, 4.5)	4.6 (3.6, 5.3)	<.0001
Vegetable protein (% of energy)	262	5.1 (4.6, 5.5)	4.8 (4.2, 5.2)	4.6 (4.1, 4.9)	0.009	5.0 (4.5, 5.5)	4.8 (4.2, 5.3)	4.6 (4.2, 5.1)	0.03
Carbohydrate (% of energy)	262	53.3 (50.0, 55.5)	50.0 (48.7, 52.9)	49.2 (47.5, 52.7)	0.001	53.6 (51.9, 56.3)	52.2 (49.7, 53.6)	48.5 (45.4, 50.0)	<.0001
Added sugar (% of energy)	262	14.9 (12.7, 19.9)	14.4 (11.9, 17.6)	12.3 (10.2, 15.3)	0.03	15.6 (12.4, 19.6)	14.7 (11.2, 18.7)	11.8 (10.0, 16.2)	0.007
Dietary GI	262	57.0 (55.3, 58.6)	56.5 (55.0, 57.7)	55.0 (52.3, 56.9)	0.001	56.1 (55.4, 57.6)	56.3 (54.3, 57.9)	55.3 (53.8, 56.4)	0.01
Dietary GL (g/ 1000 kcal	262	74.8 (72.1, 77.7)	72.0 (67.7, 74.8)	67.8 (65.1, 71.9)	<.0001	75.3 (72.0, 79.9)	73.9 (67.1, 77.1)	66.5 (63.2, 70.9)	<.0001
Dietary fiber (g/ 1000 kcal)	262	10.6 (9.1, 11.9)	9.7 (8.6, 11.0)	9.6 (8.1, 10.5)	0.007	10.9 (9.7, 12.1)	10.2 (9.0, 12.3)	10.1 (9.0, 11.6)	0.15
Calcium (mg/ 1000 kcal)	262	420 (348, 488)	487 (429, 524)	526 (420, 591)	0.0009	415 (377, 466)	472 (380, 521)	501 (428, 575)	<.0001

DONALD Study, Germany
Abbreviations: GI, glycemic index; GL, glycemic load. Values are medians (25th percentile, 75th percentile).
[a]Differences between the tertiles were tested using a Kruskal-Wallis test for continuous variables and χ^2-test for categorical variables. **P**-values ≥ 0.2 with one decimal.
Intakes of meat protein and milk protein do not add up to total animal protein consumption as fish- and egg protein are missing.

TABLE 3 Relation of dietary protein intake during puberty to fat mass index and fat-free mass index in young adulthood

	Fat mass index, FMI (kg/m²)				Fat-free mass index, FFMI (kg/m²)			
	Tertile 1	Tertile 2	Tertile 3	$p_{for\ trend}$	Tertile 1	Tertile 2	Tertile 3	p for trend
Animal protein								
Females (n = 140)	*6.4%[a]*	*7.9%*	*9.6%*		*6.4%*	*7.9%*	*9.6%*	
Model A	6.2 (5.8−6.7)	6.1 (5.7−6.5)	6.9 (6.4−7.4)	0.4	15.3 (15.0−15.6)	15.3 (15.0−15.6)	15.9 (15.6−16.2)	0.02
Model B	6.3 (5.9−6.9)	6.4 (5.9−6.9)	7.2 (6.6−7.8)	0.5	15.3 (15.0−15.5)	15.4 (15.1−15.7)	16.2 (15.9−16.6)	0.001
Males (n = 120)	*7.0%*	*8.4%*	*10.0%*		*7.0%*	*8.4%*	*10.0%*	
Model A	4.2 (3.7−4.7)	3.7 (3.3−4.2)	3.6 (3.2−4.1)	0.4	18.9 (18.5−19.3)	19.1 (18.7−19.5)	18.9 (18.5−19.3)	0.2
Model B	4.3 (3.7−5.0)	3.7 (3.2−4.2)	3.6 (3.1−4.1)	0.14	18.9 (18.4−19.3)	19.0 (18.6−19.4)	18.9 (18.5−19.4)	0.3
Plant protein								
Females (n = 140)	*4.1%*	*4.8%*	*5.5%*		*4.1%*	*4.8%*	*5.5%*	
Model A	6.3 (5.8−6.7)	6.5 (6.0−6.9)	6.4 (6.0−6.9)	0.4	15.6 (15.3−15.9)	15.7 (15.4−16.0)	15.2 (14.9−15.5)	0.13
Model B	6.4 (6.0−6.9)	6.8 (6.3−7.3)	6.7 (6.2−7.2)	0.3	15.7 (15.3−16.0)	15.8 (15.5−16.1)	15.4 (15.1−15.7)	0.3
Males (n = 122)	*4.1%*	*4.8%*	*5.5%*		*4.1%*	*4.8%*	*5.5%*	
Model A	3.9 (3.4−4.5)	3.8 (3.4−4.4)	3.7 (3.2−4.2)	0.99	19.0 (18.6−19.4)	19.0 (18.6−19.4)	18.9 (18.5−19.3)	0.9
Model B	3.8 (3.3−4.3)	4.1 (3.6−4.7)	3.6 (3.1−4.1)	0.7	18.8 (18.4−19.2)	19.2 (18.7−19.6)	18.8 (18.4−19.2)	0.97

DONALD Study, Germany
Values are least squares means and 95% confidence intervals.
Tertiles: energy-adjusted tertiles of animal protein intake during puberty (baseline).
p for trend: p for linear trend, calculated in multiple linear regression analyses.
Model A: adjusted for the respective baseline value (FMI in FMI-models and FFMI in FFMI-models) and age in young adulthood.
Model B: Model A + adjustment for early life factors (breast feeding), socioeconomic factors (maternal education status) and nutritional factors (dietary glycemic index, intakes of calcium and energy). Models for FMI were further adjusted for birth weight and maternal overweight, as well as for dietary fiber—except when considering plant protein in order to avoid multicollinearity.
[a]Median intake levels of animal protein, as % of energy.

than a substitution for fats. Moreover, it seemed to be protein from meat sources that was responsible for the results observed concerning animal protein in women. However, as the variation of dairy protein intake in our female study sample was substantially smaller than that of meat protein intake, the interpretability of this finding is limited.

Young adult FMI levels were not related to pubertal plant or animal protein consumption in females. However, the difference in mean FMI between the extreme tertiles of animal protein intake was essentially the same as for FFMI (both 0.9 kg/m²; see **Table 5**). The width of the confidence intervals, on the other hand, was much

TABLE 4 Relation of pubertal protein intake to young adult levels of FMI at fixed levels of FFMI, and vice versa

	Fat mass index, FMI (kg/m²)				Fat-free mass index, FFMI (kg/m²)			
	Tertile 1	Tertile 2	Tertile 3	$p_{for\ trend}$	Tertile 1	Tertile 2	Tertile 3	$p_{for\ trend}$
Animal protein								
Females (n = 140)	*6.4%[a]*	*7.9%*	*9.6%*		*6.4%*	*7.9%*	*9.6%*	
	6.5 (6.1−7.0)	6.5 (6.0−6.9)	6.7 (6.2−7.2)	0.3	15.3 (15.0−15.5)	15.4 (15.2−15.7)	16.0 (15.7−16.3)	0.001
Males (n = 120)	*7.0%*	*8.4%*	*10.0%*		*7.0%*	*8.4%*	*10.0%*	
	4.5 (4.0−5.1)	3.6 (3.2−4.1)	3.4 (3.0−3.8)	0.001	18.7 (18.3−19.0)	19.0 (18.7−19.4)	19.0 (18.7−19.4)	0.04
Plant protein								
Females (n = 140)	*4.1%*	*4.8%*	*5.5%*		*4.1%*	*4.8%*	*5.5%*	
	6.5 (6.1−7.0)	6.5 (6.0−6.9)	6.6 (6.2−7.1)	0.2	15.6 (15.3−15.9)	15.8 (15.5−16.0)	15.3 (15.1−15.6)	0.09
Males (n = 122)	*4.1%*	*4.8%*	*5.5%*		*4.1%*	*4.8%*	*5.5%*	
	3.8 (3.4−4.3)	4.0 (3.5−4.5)	3.6 (3.2−4.1)	0.9	18.9 (18.5−19.2)	19.0 (18.6−19.4)	18.9 (18.5−19.2)	0.7

DONALD Study, Germany
Values are least squares means and 95% confidence intervals.
Tertiles: energy-adjusted tertiles of animal protein intake during puberty (baseline).
p for trend: p for linear trend, calculated in multiple linear regression analyses.
FMI-models: adjusted for FFMI in young adulthood, baseline- fat mass index, age in young adulthood, breast feeding, birth weight, maternal overweight, maternal education status, glycemic index, intakes of fiber, calcium and energy. When considering plant protein, there was no adjustment for fiber in order to avoid multicollinearity.
FFMI-models: adjusted for FMI in young adulthood, baseline- fat-free mass index, age in young adulthood, breast feeding, maternal education status, glycemic index, intakes of calcium and energy.
[a]Median intake levels of animal protein, as % of energy.

141

bigger for FMI than for FFMI. Thus, insufficient statistical power resulting from the relatively high variance of FMI (34) and from our relatively small sample sizes must be considered as one cause for the absence of significant results concerning FMI.

In men, we only observed relations between animal protein intake and adult FFMI or FMI when holding levels of the respective complementary component of body composition (adult FMI, FFMI) constant. The emergence of significant associations after vice versa adjustment is probably because of the opposing associations (i.e. the fact that higher pubertal animal protein intakes tended to be related to lower adult FMI and higher adult FFMI levels) as well as the marked correlation of adult FMI and FFMI levels (r = 0.61 among males and r = 0.55 in females). The public health relevance of these findings is not straightforward, as holding one part of body mass constant makes it difficult to consider body composition as the entity that it is in reality. However, the results are of interest from a mechanistic point of view. In men, the difference in FMI between low and high pubertal animal protein consumption (absolute difference between the first and the third tertile, Table 4: 1.1 kg/ m^2) was notably larger than the respective difference in FFMI (absolute difference, Table 4: 0.3 kg/ m^2), indicating that among males variations in pubertal animal consumption may primarily affect adult fat mass. Of note, this contrasts to our findings for females, in whom a higher pubertal animal consumption appeared to primarily affect adult fat-free mass. It is imaginable that, in boys, hormonal influences on the construction of muscle mass are much more important than variations in protein consumption within the range of usual intakes.

We conducted additional analyses that revealed that besides puberty, the period of adiposity rebound, but not early childhood, may be relevant for the long-term development of body composition: Among males, we observed a positive relation between animal protein intake during the period of adiposity rebound and FFMI in young adulthood with borderline significance ($p = 0.05$). However, our analyses conducted for the early life and the adiposity rebound period are limited by small sample sizes and must hence be interpreted with caution.

One physiological mechanism by which higher intakes of animal protein could increase FFMI is an anabolic effect of essential amino acids (EAA) on muscle mass, which has been observed in small experimental studies among younger and older adults (25). A suggested biochemical pathway for the stimulation of muscle protein synthesis by the EAA leucine is the activation of the protein kinase mammalian target of rapamycin (mTOR) and its downstream effectors eukaryotic initiation factor 4E (EIF4E) and ribosomal S-6 kinase (S6K1) (26). Given the generally lower EAA content of plant protein compared to animal protein (27), our finding that plant protein intake was not associated with FFMI, neither in males nor in females, is quite plausible. Still, in order to verify that such a relation was not only masked because of a weak inverse correlation of plant protein and animal protein consumption in our sample ($r = 0.27$), we ran additional analyses in which we adjusted models examining plant protein intake for animal protein (data not shown). However, such an adjustment did not notably change our results.

RCTs conducted among a pubertal or young adult population provide evidence for a role of animal protein from different sources as stimulants of fat-free mass increase: An RCT with 6-14 year old Kenyan children showed an effect of a meat supplementation on mid-upper-arm muscle area, but not mid-upper-arm fat area (28). In an RCT with 98 eight to ten year old Chilean girls, replacement of sugar-sweetened beverages for milk for 16 weeks yielded an additional gain in fat-free mass, but not fat mass, in comparison with the control group (29). In three other intervention studies with pubertal or young adult subjects, a supplementation with milk products had no significant effect on body composition (30-32); however, these studies were not specifically designed to investigate changes in body composition and had smaller sample sizes. Hence, our main finding of a prospective relation between animal protein during puberty and FFMI in young adulthood among women is generally consistent with evidence from RCTs.

The findings from other epidemiologic studies with pubertal or young adult study populations concerning a link between protein intake and fat mass are mixed. In a Danish cohort study with 350 participants, higher protein intakes in puberty were related to a higher percentage body fat in young adulthood among women (35), and in an American cohort study with 2909 participants, higher protein intakes in young adulthood were prospectively related to a slightly higher waist-to-hip-ratio among white participants (7). On the other hand, in a Dutch epidemiologic study with 364 subjects, higher intakes of protein were prospectively associated with a lower FMI among leaner girls, and with a higher FFMI among girls in the 5th BMI-quintile (36).

In terms of public health relevance, our study does not suggest a strong unfavorable effect of relatively high animal protein intake levels on body composition. Even under the assumption that a larger sample size would render the results for FMI seen in women significant, the effect sizes seen in this study do not imply a disproportionally higher increase in FMI than in FFMI. On the other hand, even in the highest energy-adjusted tertile of animal protein intake, protein accounted for less than 15% of energy intake (see **Table** 2. Still higher intake levels could affect body composition in a different manner.

Limitations of our study include, first of all, its observational design. We cannot exclude that our results might be biased by residual confounding. Additionally, as observational studies need energy-adjustment to limit the impact of confounding and underreporting, potential satiety-mediated effects of protein (38) can be shown less well than in intervention studies under ad-libitum conditions.

Second, we determined FMI and FFMI on the basis of skinfold thickness measurements, which have a higher susceptibility to measurement error than specialized research methods such as hydrodensitometry, magnetic resonance imaging, and BodPod. Yet, the skinfold equations of Durnin and Womersley (19) agree, on average, very well with the results from hydrodensitometry (39). In addition, measurements were performed by trained and quality-monitored personnel, which has been shown to notably reduce intra- and interobserver variability (40). It would have been interesting to specifically consider abdominal fat mass as a body fat distribution characterized by high intra-abdominal fat is known to be particularly detrimental. However, in our sample, there was not enough data available to conduct such analyses. Third, DONALD participants are characterized by a relatively high socio-economic status (14) and only 20.6% of the participants study sample were overweight in young adulthood. It is possible that the relative homogeneity of our sample means that extremes of diet and behavior are not represented. Forth, we merely disposed a crude measure of physical activity at the age of five years derived from parental questionnaires, available for 198

participants of our study sample. When we repeated our analyses with further adjustment for physical activity in this subgroup, we obtained essentially the same results as in our main analyses. The main strengths of our study are its prospective nature and the carefully collected, repeated data on growth and diet, covering the entire time of childhood and adolescence. The availability of data on several potential confounders, such as parental characteristics, is an additional strength of our analysis.

Conclusion

In conclusion, our results indicate that, particularly among females, a habitually higher consumption of animal protein during puberty yields a higher FFMI in young adulthood. This argues against a selective increase in fat mass as a consequence of relatively high intake levels of animal protein. O

Acknowledgments

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143

Animal Protein Intakes during Early Life and Adolescence Differ in Their Relation to the Growth Hormone-Insulin-Like-Growth-Factor Axis in Young Adulthood[1,2]

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Abstract

Recent studies provide evidence that insulin-like-growth-factor I (IGF-I) and its binding proteins (IGFBP) IGFBP-2 and IGFBP-3 are related to the risk of several common cancers. It remains to be clarified whether their concentrations can be programmed by protein intake from different sources during growth. This study addressed the hypothesis that animal protein intakes during infancy, mid-childhood, and adolescence differ in their relevance for the growth-hormone (GH)-IGF-I axis in young adulthood. Data from the Dortmund Nutritional and Anthropometric Longitudinally Designed Study participants with at least 2 plausible 3-d weighed dietary records during adolescence (age: girls, 9–14 y; boys, 10–15 y; $n = 213$), around the adiposity rebound (age 4–6 y; $n = 179$) or early life (age 0.5–2 y; $n = 130$), and one blood sample in young adulthood were included in the study. Mean serum concentrations of IGF-I, IGFBP-1, IGFBP-2, and IGFBP-3 were compared between tertiles of habitual animal protein intake using multivariable regression analysis. Habitually higher animal protein intakes in females during puberty were related to higher IGF-I (P-trend = 0.005) and IGFBP-3 (P-trend = 0.01) and lower IGFBP-2 (P-trend = 0.04), but not to IGFBP-1 in young adulthood. In turn, IGF-I concentrations in young adulthood were inversely related to animal protein intakes in early life among males only (P-trend = 0.03), but not to animal protein intake around adiposity rebound (P-trend > 0.5). Our data suggest that, among females, a habitually higher animal protein intake during puberty may precipitate an upregulation of the GH-IGF-I axis, which is still discernible in young adulthood. By contrast, among males, higher animal protein intakes in early life may exert a long-term programming of the GH-IGF-I axis. *J. Nutr.* 143: 1147–1154, 2013.

Introduction

The growth hormone (GH)[10]-insulin-like-growth-factor I (IGF-I) axis plays a central role in cell proliferation and apoptosis (1) and has been related to different cancers (2,3). The bioavailability of IGF-I is determined by its binding proteins (IGFBP), with IGFBP-1 and IGFBP-3 limiting its acute and longer term

bioavailability. Furthermore, IGFBP-3 and IGFBP-2 concentrations are inversely associated with cellular proliferation and apoptosis independently of IGF-I (4,5), whereas lower IGFBP-2 concentrations are also considered to reflect a lower long-term insulin sensitivity (4).

Several recent cross-sectional studies in adults suggest that high protein intakes, particularly animal protein, are related to higher IGF-I (6–8) and IGFBP-3 concentrations (7) and that the source of animal protein is an important determinant of IGF-I levels, with most studies pointing to dairy products or milk (6,7,9) and others to meat (8,10).

The IGF system has a central role in the regulation of fetal and childhood growth and metabolism (11). Hence, it is plausible to assume that protein intake in childhood in particular may be related to the GH-IGF-I axis. Indeed, a number of intervention studies in children showed a relation between higher

[10] Abbreviations used: DONALD, Dortmund Nutritional and Anthropometric Longitudinally Designed; FFMI, fat-free mass index; GH, growth hormone; IGFBP, insulin-like-growth-factor binding protein; IGF-I, insulin-like-growth-factor I.
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1147

milk intake and higher IGF-I (12–14) and IGFBP-3 (13) concentrations. However, not all studies confirmed the association with IGF-I concentrations (14,15). Only 2 prospective studies have addressed the long-term relevance of (animal) protein intake during growth and/or its sources in relation to the GH-IGF-I axis. Ben-Shlomo et al. (16) and Martin et al. (17) found an inverse association between milk intakes in early childhood and IGF-I concentrations in young adulthood (16) and older age (17). The authors proposed that this inverse relation reflects an early programming of the GH-IGF-I axis in response to higher (animal) protein intakes in early life. Hence, early pituitary resetting in response to higher ambient IGF-I concentrations may occur, which would ultimately result in an inverse association between animal protein in early life and IGF-I concentrations in young adulthood. However, prospective evidence covering different, potentially critical, developmental periods is lacking to unravel whether such an inverse association between animal protein intake and the GH-IGF-I axis is confined to early life.

The main hypothesis of this study is that consumption of animal protein and its components (i.e., meat and dairy protein) during puberty, a period when the GH-IGF-I axis undergoes major changes, is of prospective relevance for IGF-I and its binding proteins in young adulthood. Because a reversal in the long-term relation between animal protein and the GH-IGF-I axis has been proposed, an additional aim was to examine whether animal protein intakes in early life or around the adiposity rebound are inversely related to IGF-I concentrations in young adulthood.

Methods

Study population. The Dortmund Nutritional and Anthropometric Longitudinally Designed (DONALD) Study is an ongoing, open cohort study conducted at the Research Institute of Child Nutrition in Dortmund, Germany. Details on this study were previously described (18). In brief, since recruitment began in 1985, detailed data on diet, growth, development, and metabolism from infancy to adulthood have been collected from >1300 healthy children. Every year, a mean of 40 infants are newly recruited and first examined at the age of 3 mo. Each child returns for 3 more visits in the first year, 2 in the second, and then once annually until adulthood. Since 2005, participants ≥18 y of age are invited for subsequent examinations with fasting blood withdrawal. The study was approved by the Ethics Committee of the University of Bonn and all examinations are performed with parental and adult participants' consent.

Because of the open cohort design, many participants have not yet reached young adulthood. Among those who did, age varied from 18 to 36 y. For 308 participants who were term (36–43 wk gestation) singletons with a birth weight ≥2500 g, one measurement of IGF-I and IGFBP-3 was available. Of these, 222 participants had provided at least 2 plausible 3-d weighed dietary records during adolescence (age: girls, 9–14 y; boys, 10–15 y), describing habitual dietary intake during puberty. Participants who had consistently underreported energy intake during puberty (i.e., all food records were implausible or they had provided more implausible than plausible food records) were excluded from the analysis (n = 18). A 3-d weighed dietary record was considered plausible when the total recorded energy intake was adequate in relation to the estimated BMR using modified age-dependent cutoffs from Goldberg et al. (19). For boys and girls aged 14 y and older, a ratio between reported energy intake and basal metabolic rate <1.07 and 0.97, respectively, was considered implausible. For boys and girls younger than 14 y, the cutoffs were 1.04 and 1.01, respectively (20). Furthermore, participants had to have anthropometric data available at the beginning of puberty and young adulthood and information on relevant covariates such as early life and socioeconomic factors. This resulted in final samples of 213 (55.4% females). Overall analyses are based on 1131 records (i.e., 2–6 records/participant; mean = 5).

1148   Joslowski et al.

Among participants with a blood sample available in young adulthood, 130 and 179 had provided a minimum of 2 plausible, 3-d weighed dietary records during early life (age 0.5–2 y) and around adiposity rebound (age 4–6 y), respectively. IGFBP-1 and IGFBP-2 measurements were missing for a few individuals, resulting in slightly lower sample sizes for these outcomes (see tables).

Clinical measurements and calculations. Venous blood samples were drawn after an overnight fast. Blood samples were frozen at −80°C and then shipped to the Laboratory for Translational Hormone Analytics in Pediatric Endocrinology at the University of Giessen. Serum samples were analyzed for IGF-I and IGFBP-3 using an RIA according to Blum et al. (21) and for IGFBP-2 and IGFBP-1 with an ELISA (Mediagnost, Germany, lot 061010 and lot 050910), respectively.

Anthropometric measurements and calculations. Participants are measured at each visit according to standard procedures with the participants dressed in underwear only and barefoot. From the age of 2 y onward, standing height is measured to the nearest 0.1 cm using a digital stadiometer (Harpenden). Body weight is measured to the nearest 100 g using an electronic scale (Seca 753E; Seca Weighing and Measuring Systems). Skinfold thicknesses are measured from the age of 6 mo onward at 4 different sites (supra-iliacal, subscapular, biceps, and triceps) on the right side of the body to the nearest 0.1 mm using a Holtain caliper (Holtain). The 3 trained nurses who perform the measurements undergo an annual quality control conducted in 6–8 healthy young adult volunteers.

Sex- and age-independent SD scores were calculated for BMI (kg/m^2) at baseline using the German reference curves for BMI (22). Percentage body fat was derived using equations of Slaughter et al. (23) for pubescent children, which includes triceps and subscapular skinfolds. From this, fat mass index and fat-free mass index (FFMI) were calculated as weight × percentage body fat/height^2 and [weight − weight × percentage body fat]/height^2, respectively.

Nutritional assessment. Food consumption in the DONALD Study is assessed annually using 3-d weighed dietary records. All foods and beverages as well as leftovers consumed are weighed and recorded to the nearest 1 g for 3 d using electronic food scales (initially Soehnle Digita 8000, Leifheit; now WEDO digi 2000, Werner Dorsch). For this analysis, dietary variables were calculated as individual means of the 3-d weighed dietary records using LEBTAB (18), the in-house database that is continuously updated to include all recorded food items. LEBTAB is based on the German standard food tables (24) and data obtained from commercial food products (25). With regard to breastfeeding, test weighing is performed (i.e., weighing the infant before and after each meal) to the nearest 10 g with the use of an infant-weighing scale (Soehnle multina 8300) (25). In this analysis, 5% was added to the test weighing results to account for insensible water losses (26).

To examine food groups providing animal protein in more detail, all recorded foods were assigned to the respective food groups, i.e., meat products, dairy products, and miscellaneous. Animal protein did not include protein from human milk.

Statistical analysis. All statistical analyses were carried out using SAS procedures (version 9.1.3, SAS Institute). *P* < 0.05 was considered significant.

Baseline characteristics are presented in sex-specific and energy-adjusted tertiles of dietary animal protein intake (T1--T3). Tests for differences were performed across the tertiles of dietary animal protein intake using ANOVA for normally distributed continuous variables, Kruskal-Wallis test for non-normally distributed continuous variables, chi-square test for categorical variables, and Fisher's exact test for categorical variables if 50% of cells had expected counts less than frequencies <5.

Multiple linear regression analysis was used to analyze the potential relation of dietary animal intake during puberty to concentrations of IGF-I, IGFBP-1, IGFBP-2, and IGFBP-3 in young adulthood. Because the outcome variables were not normally distributed, IGF-I and IGFBP-2 were transformed prior to analysis using the square root, and IGFBP-1 and

TABLE 1 Demographic, anthropometric, birth, and socioeconomic characteristics by energy-adjusted tertiles of animal protein during puberty (*n* = 213) (DONALD Study, Germany)[1]

	Males			Females		
	T1	T2	T3	T1	T2	T3
Animal protein intake, *g/d*	38.4 (30.7, 46.4)	42.9 (36.9, 49.4)	49.8 (46.9, 57.6)*	26.2 (21.8, 31.7)	32.8 (30.9, 36.2)	41.6 (37.8, 48.7)*
All, *n*	31	32	32	39	40	39
Age, *y*	10.00 (9.97, 10.18)	10.02 (9.98, 10.09)	9.99 (9.97, 10.05)	8.99 (8.97, 9.02)	9.03 (8.98, 9.07)	8.99 (8.98, 9.07)
BMI-SDS	−0.27 ± 0.93	0.13 ± 0.83	0.34 ± 0.59*	−0.27 ± 0.95	−0.16 ± 0.88	0.28 ± 0.97*
BMI, *kg/m²*	15.7 (15.3, 18.8)	17.7 (16.1, 18.8)	18.0 (16.8, 18.7)	15.7 (15.0, 17.2)	15.9 (15.0, 17.4)	17.6 (15.4, 19.0)*
FMI,[2] *kg/m²*	2.0 (1.6, 3.7)	3.0 (2.0, 4.4)	2.7 (2.3, 3.2)	2.6 (2.2, 3.3)	2.7 (2.2, 3.6)	3.7 (2.3, 4.6)
FFMI,[3] *kg/m²*	13.8 (13.3, 14.6)	14.3 (13.3, 15.2)	15.2 (14.1, 15.8)*	13.2 (12.6, 13.9)	13.3 (12.6, 14.0)	13.7 (13.0, 14.7)*
Birth weight, *g*	3558 ± 403	3487 ± 461	3549 ± 470	3490 ± 489	3395 ± 393	3381 ± 425
Birth length, *cm*	53 (52, 54)	52 (50, 53)	52 (50, 54)	52 (50, 53)	51 (50, 53)	51 (49, 52)
Pregnancy duration, *wk*	40 (39, 41)	40 (39, 40)	40 (39, 40)	40 (40, 41)	40 (40, 41)	40 (39, 40)
Birth weight and length appropriate for gestational age,[4] *n (%)*	25 (80.6)	23 (71.9)	27 (84.4)	29 (74.4)	35 (87.5)	29 (74.4)
Breast feeding >2 wk,[5] *n (%)*	23 (74.2)	20 (62.5)	22 (68.8)	29 (74.4)	29 (72.5)	26 (66.7)
Maternal overweight,[6] *n (%)*	7 (22.6)	8 (25.0)	14 (43.8)	10 (25.6)	11 (27.5)	17 (43.6)
Maternal education,[7] *n (%)*	16 (51.6)	9 (28.1)	21 (65.6)*	21 (53.8)	22 (55.0)	12 (30.8)
Maternal employment,[8] *n (%)*	15 (48.4)	14 (43.8)	19 (59.4)	18 (46.2)	24 (60.0)	17 (43.6)
Paternal overweight,[6] *n (%)*	12 (52.2)	12 (48.0)	20 (74.1)	16 (50.0)	20 (55.6)	14 (45.2)
Paternal education,[7] *n (%)*	16 (55.2)	15 (46.9)	17 (53.1)	22 (59.5)	22 (57.9)	14 (36.8)
Paternal employment,[8] *n (%)*	28 (96.6)	30 (93.8)	29 (90.6)	35 (94.6)	38 (100)	37 (97.4)
Smokers in the household, *n (%)*	6 (19.4)	17 (53.1)	10 (31.3)*	14 (35.9)	17 (42.5)	9 (23.1)

[1] Values are means ± SDs or median (25th and 75th percentiles). Differences between the tertiles were tested using ANOVA for normally distributed continuous variables, Kruskal-Wallis test for not normally distributed continuous variables, chi-square test for categorical variables, and Fisher's exact test for categorical variables if 50% of cells had expected counts less than frequencies <5. *$P < 0.05$ for differences between tertiles. DONALD, Dortmund Nutritional and Anthropometric Longitudinally Designed; FMI, fat mass index; FFMI, fat-free mass index; SDS, SD score; T, tertile.
[2] FMI calculated as weight × percentage of body fat/height².
[3] FFMI calculated as [weight − weight × percentage of body fat)]/height².
[4] That is, birth weight and length were between the 10th and 90th percentiles of the German sex-specific birth weight-for-gestational-age curves (28).
[5] Breast feeding categories: ≤2 wk or >2 wk of full breastfeeding.
[6] Maternal and paternal BMI ≥25 kg/m²; paternal BMI: *n* = 39 missing.
[7] School education for at least 12 y; paternal education: *n* = 7 missing.
[8] Maternal and paternal employment (yes/no); paternal employment: *n* = 7 missing.

IGFBP-3 were log-transformed to obtain normal distribution. Back-transformed data are presented for ease of interpretation. All dietary variables were energy adjusted using the residual method (27). To account for age-dependent changes in intake, all variables were standardized by age group and sex (mean = 0, SD = 1).

For this analysis, the following covariates were considered as potentially confounding factors: birth weight and length appropriate for gestational [yes/no, defined as birth weight and birth length between the 10th and 90th percentiles of the German sex-specific birth weight and height-for-gestational age curves (28)], full breastfeeding for >2 wk (yes/no), maternal or paternal overweight status (BMI ≥25 kg/m², yes /no), high maternal or paternal educational status (≥12 y of schooling, yes /no), maternal or paternal occupation (whether parents were employed, yes/no), smokers in the household (yes/no), and FFMI at the beginning of puberty. The basic model considers age in young adulthood only (model A). In the next step, each potential confounder was initially considered separately, yet only covariates that substantially affected the associations between animal protein intakes and parameters of the GH-IGF-axis (by ~10% or more) were included in model B. To explicitly assess the effect of substituting animal for plant protein, we ran an additional model that included total energy and all energy-bearing macronutrients except plant protein (i.e., total fat, total carbohydrate, animal protein in percent energy). The coefficient (β) obtained for animal protein then reflects the effect of substituting animal for plant protein, because total energy, fat, and carbohydrates are held constant (27). Similar models were run to address substitution of animal protein for carbohydrates or fats.

All analyses were stratified by sex based on the following considerations: *1)* the association between animal protein intake during puberty and our main outcome, i.e., IGF-I concentrations, differed between males and females (*P*-interaction < 0.1); *2)* stratified analyses revealed

that the relevance of the investigated exposure-outcome relations consistently differed between genders; and *3)* both growth and IGF-I concentrations are known to differ between genders (29,30), supporting the biological plausibility of stratification. A similar approach was used to analyze the potential relation of dietary animal protein intake in early life and around adiposity rebound to IGF-I concentrations in young adulthood. All models conform to the assumptions of linear regression models (linearity, normality and homoscedasticity of residuals, absence of multicollinearity).

Results

The characteristics of participants in this study at the beginning of puberty are presented in tertiles of dietary animal protein intake during puberty (**Table 1**). Females and males in the highest tertile of dietary animal protein intake were more likely to have had a higher BMI-SD score and a higher FFMI at the beginning of puberty. Males in the middle tertile of dietary animal protein intake were least likely to have a mother with a high educational level and most likely to live in a household with smokers (Table 1).

By definition, higher animal protein intakes were related to higher total protein, meat, and dairy intakes, but not to plant protein. Higher animal protein intakes in both males and females were also related to lower carbohydrate intakes, but not to fiber intakes. Of note, higher intakes of animal protein were associated with higher intakes of total fat and MUFAs in females only (**Table 2**).

Dietary protein sources and GH-IGF-I axis 1149

TABLE 2 Nutritional data during puberty by energy-adjusted tertiles of animal protein intake during puberty (n = 213) (DONALD Study, Germany)[1]

	Males			Females		
	T1	T2	T3	T1	T2	T3
Animal protein intake, g/d	38.4 (30.7, 46.4)	42.9 (36.9, 49.4)	49.8 (46.9, 57.6)*	26.2 (21.8, 31.7)	32.8 (30.9, 36.2)	41.6 (37.8, 48.7)*
All, n	31	32	32	39	40	39
Total energy, MJ/d	9.1 (8.1, 10.5)	8.62 (7.3, 9.6)	8.8 (8.2, 9.7)	7.3 (6.4, 8.1)	7.1 (6.3, 7.6)	7.2 (6.5, 8.1)
Fat, %en	35.3 ± 4.3	35.9 ± 3.5	35.8 ± 3.5	34.9 ± 3.9	35.7 ± 3.5	37.5 ± 4.3*
SFA, %en	15.5 ± 2.6	16.0 ± 1.9	15.5 ± 1.5	15.7 ± 2.2	15.9 ± 1.9	16.4 ± 2.6
PUFA, %en	5.3 ± 1.3	5.1 ± 0.9	5.2 ± 1.2	5.2 ± 1.1	5.2 ± 0.9	5.6 ± 1.1
MUFA, %en	10.9 ± 1.3	11.2 ± 1.4	11.5 ± 1.4	10.6 ± 1.6	11.1 ± 1.3	11.8 ± 1.6*
Protein, %en	11.8 ± 1.1	13.2 ± 0.8	14.5 ± 0.9*	11.2 ± 0.9	12.8 ± 0.8	14.5 ± 1.2*
Animal protein, %en	7.0 ± 0.9	8.3 ± 0.4	9.8 ± 0.9*	6.2 ± 0.9	7.9 ± 0.4	9.8 ± 1.0*
Meat protein, %en	2.7 (2.0, 3.2)	3.1 (2.6, 3.9)	3.5 (2.7, 5.4)*	1.8 (1.2, 2.3)	3.2 (2.2, 4.1)	3.9 (2.7, 4.6)*
Dairy protein, %en	3.5 ± 1.2	4.4 ± 0.7	4.6 ± 1.3*	3.5 ± 0.9	3.7 ± 1.1	4.6 ± 1.4*
Plant protein, %en	4.9 ± 0.8	4.8 ± 0.8	4.6 ± 0.7	5.0 ± 0.7	4.9 ± 0.7	4.7 ± 0.7
Consumers of alcohol, n (%)	5 (16.1)	2 (6.3)	2 (6.3)	2 (5.1)	1 (2.5)	3 (7.7)
Carbohydrate, %en	52.9 ± 4.3	50.9 ± 3.6	49.7 ± 3.5*	53.9 ± 4.1	51.5 ± 3.3	48.0 ± 4.4*
Added sugar, %en	16.8 ± 5.6	14.8 ± 4.6	13.9 ± 3.9	16.4 ± 4.9	15.1 ± 4.2	12.6 ± 4.6*
Fiber, g/d	21.3 (17.2, 26.9)	20.1 (17.6, 22.5)	20.6 (16.3, 24.4)	17.8 (16.2, 21.2)	16.8 (15.4, 19.9)	18.4 (16.0, 19.8)

[1] Values are means ± SDs or median (25th and 75th percentiles). Differences between the tertiles were tested using ANOVA for normally distributed continuous variables, Kruskal-Wallis test for not normally distributed continuous variables, and Fisher's exact test for categorical variables if 50% of cells had expected counts less than frequencies under 5. *$P < 0.05$ for differences between tertiles. DONALD, Dortmund Nutritional and Anthropometric Longitudinally Designed; T, tertile; %en, percent energy.

Puberty. Among females, a habitually lower animal protein intake during puberty was associated with lower concentrations of IGF-I and IGFBP-3, but not with IGFBP-1 (**Table 3**, model A). Similar results were seen after additional adjustment for socioeconomic factors and FFMI at the beginning of puberty (Table 3, model B). Conversely, a higher animal protein intake was related to lower levels of IGFBP-2 (model B). Animal protein was not related to IGF-I, IGFBP-3, IGFBP-1, or IGFBP-2 in males.

Substitution models revealed similar associations for a substitution of animal protein intake for total fat (β animal protein = 0.5137; P-trend = 0.01) or total carbohydrate intake (β animal protein = 0.4812; P-trend = 0.03) with respect to its association with IGF-I concentrations. Substitution of animal protein intake for plant protein intake was related to slightly lower, albeit nonsignificant increases in IGF-I concentrations (β animal protein = 0.4131; P-trend = 0.4).

Analysis of animal protein sources revealed that intake of dietary meat protein only was significantly associated with adult IGF-I concentrations (**Fig. 1**A). No associations were found in males for any protein source. Plant protein was not related to the IGF-I axis among females or males (data not shown).

Additional consideration of early-life characteristics (e.g., birth weight, full breastfeeding) or other nutritional variables (e.g., monounsaturated fat or plant protein intake) did not affect the results (data not shown).

TABLE 3 Relation of dietary animal protein intake during puberty to serum IGF-I, IGFBP-1, IGFBP-2, and IGFBP-3 concentrations in young adulthood (DONALD Study, Germany)[1]

	Males				Females			
	T1	T2	T3	P-trend	T1	T2	T3	P-trend
Animal protein intake,[2] g/d	38.4 (30.7, 46.4)	42.9 (36.9, 49.4)	49.8 (46.9, 57.6)	<0.0001	26.2 (21.8, 31.7)	32.8 (30.9, 36.2)	41.6 (37.8, 48.7)	<0.0001
IGF-I, $g \cdot L^{-1}$								
Model A	237 (210, 267)	266 (237, 297)	218 (192, 245)	0.7	213 (185, 244)	268 (236, 302)	241 (211, 273)	0.01
Model B	242 (212, 273)	261 (232, 293)	222 (194, 253)	0.8	204 (177, 232)	261 (231, 292)	241 (209, 275)	0.005
IGFBP-1, $\mu g \cdot L^{-1}$								
Model A	6.5 (4.4, 9.6)	4.1 (2.8, 6.0)	6.6 (4.5, 9.7)	0.7	12.6 (9.4, 17.0)	7.8 (5.9, 10.5)	9.92 (7.40, 13.30)	0.7
Model B	5.4 (3.6, 8.1)	4.4 (3.0, 6.4)	5.6 (3.7, 8.3)	0.6	12.3 (9.1, 16.7)	7.8 (5.8, 10.4)	10.1 (7.31, 13.95)	0.9
IGFBP-2, $\mu g \cdot L^{-1}$								
Model A	193 (158, 232)	185 (151, 222)	219 (182, 260)	0.9	155 (130, 184)	136 (112, 162)	123 (100, 149)	0.09
Model B	183 (145, 225)	175 (139, 214)	224 (183, 268)	0.8	155 (128, 183)	130 (106, 157)	113 (90, 138)	0.04
IGFBP-3, $mg \cdot L^{-1}$								
Model A	3.3 (3.0, 3.6)	3.2 (2.9, 3.5)	3.5 (3.1, 3.8)	0.4	3.5 (3.3, 3.8)	3.6 (3.3, 3.8)	3.7 (3.4, 4.0)	0.02
Model B	3.3 (2.9, 3.6)	3.2 (2.9, 3.6)	3.3 (3.0, 3.7)	>0.9	3.5 (3.3 3.8)	3.5 (3.3, 3.8)	3.7 (3.4, 4.0)	0.01

[1] Values are means and 95% CIs unless otherwise indicated; n = 213. For IGF-I and IGFBP-3: 118 females/95 males; for IGFBP-1 and IGFBP-2: 109 females/92 males. Model A: adjusted for age in adulthood. Model B for IGF-I, IGFBP-1, and IGFBP-3: model A + socioeconomic factors (maternal education, smokers in the household) and FFMI at the beginning of puberty. Model B for IGFBP-2: model A + socioeconomic factors (maternal education, maternal overweight) and FFMI at the beginning of puberty. P-trend refers to the P value obtained in linear regression models with dietary animal protein as continuous variable. DONALD, Dortmund Nutritional and Anthropometric Longitudinally Designed; FFMI, fat-free mass index; IGF-I, insulin-like-growth-factor I; IGFBP, insulin-like-growth-factor binding protein; T, tertile.
[2] Values are medians (25th and 75th percentiles).
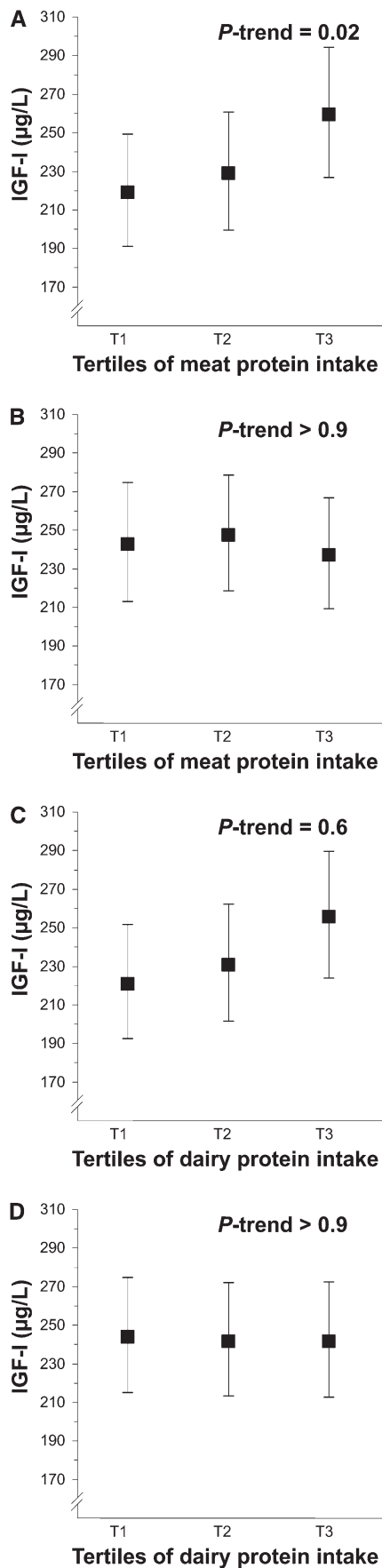
Early life (age 0.5–2 y). Among females, animal protein was not related to IGF-I (**Fig. 2***A*). Among males, a habitually higher animal protein intake in early life was associated with lower concentrations of IGF-I in young adulthood, after controlling for early life and socioeconomic factors (Fig. 2*B*). Plant protein intake in early life was not associated with IGF-I levels in young adulthood, neither among males nor among females (data not shown). Again, additional consideration of other nutritional variables (e.g., total energy intake) did not affect the results (data not shown).

Adiposity rebound (age 4–6 y). No associations were seen between animal protein intake around adiposity rebound and IGF-I in young adulthood in either females or males (Fig. 2*C,D*). Similarly, there was no association with plant protein (data not shown).

Discussion

This study provides epidemiological evidence for a prospective association between a habitually higher animal protein intake during puberty and higher concentrations of IGF-I and IGFBP-3 as well as lower IGFBP-2 concentrations in females only, suggesting an upregulation of the GH-dependent components of the GH-IGF-I axis in young adulthood. By contrast, higher animal protein intakes in early life may yield a long-term downregulation of the GH-IGF-I axis in males.

Potential mechanisms by which a higher animal protein intake may contribute to higher IGF-I levels may relate to specific amino acids, e.g., arginine (31) or combinations of amino acids (lysine and arginine) (32), which increase GH concentrations and lead to an increase in hepatic IGF-I production. Dairy and meat both contain glutamine, lysine, and arginine, but their concentrations are ~10 times higher in meat than in milk. Alternatively, it has been proposed that components in milk itself rather than animal protein as such stimulates IGF-I secretion (33). Most intervention studies in infants (34), children (12–15), and also adults (35,36) suggest that milk and dairy products are important upregulators of IGF-I concentrations. However, some (8,10) but not all (6,7,9) cross-sectional studies in adults report associations of a higher consumption of red meat with higher IGF-I concentrations. Our study supports a relevance of animal protein in general rather than dairy protein intake per se. In addition, among females, the difference between the intake levels in the lowest and highest tertiles was higher for meat protein (12.4 g) than for dairy protein (9.6 g), which may partly explain why we found an association with meat but not dairy protein. Finally, our substitution analyses revealed broadly similar effect sizes when simulating substitutions of animal protein for carbohydrate, fat, or plant protein.

We furthermore observed lower IGFBP-2 and higher IGFBP-3, but not higher IGFBP-1 concentrations, in young adulthood among females who had consumed more animal protein during

FIGURE 1 Relations of dietary meat (*A,B*) and dairy protein (*C,D*) intake during puberty to IGF-I in young adulthood among 118 females (*A,C*) and 95 males (*B,D*) in the DONALD Study, Germany. Data are means (95% CI) adjusted for age in adulthood, socioeconomic factors (maternal education, smokers in the household), and FFMI at the beginning of puberty. *P*-trend refers to the *P* value obtained in linear regression models with dietary meat or dairy protein as the continuous variable. DONALD, Dortmund Nutritional and Anthropometric Longitudinally Designed; FFMI, fat-free mass index; IGF-I, insulin-like-growth-factor I; T, tertile.
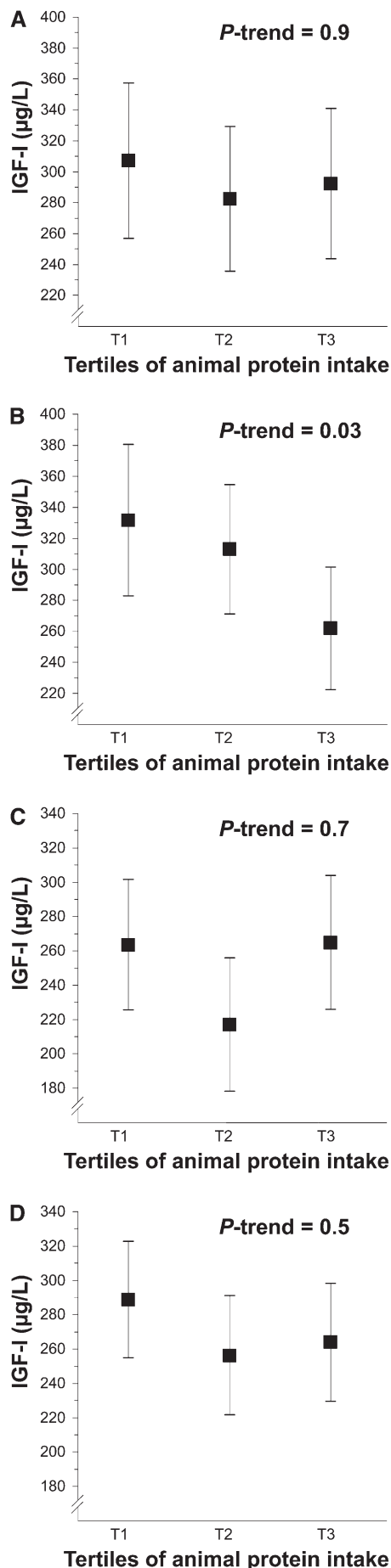
puberty. In contrast to the most abundant IGFBP-3, IGFBP-1 concentrations are responsive to acute dietary stimuli (37). The fact that the association with pubertal animal protein intake was confined to adult IGFBP-3 concentrations hence supports the underlying hypothesis of long-term nutritional influences on the adult GH-IGF-axis. Lower IGFBP-2 concentrations in turn are considered to also reflect a lower insulin sensitivity (4). In line with this, we also observed a tendency toward higher HOMA levels among females with a higher animal protein intake (P-trend = 0.1; data not shown). In addition, prospective cohort studies in adults suggest that a higher consumption of total protein as well as animal protein or red and processed meat is related to an increased risk of type 2 diabetes (38,39). One explanation for a corresponding increase in insulin resistance could be an amino acid-induced upregulation of the serine kinase 6–1 pathway, which has been shown to result in lower insulin sensitivity (40). A lower insulin sensitivity may in turn decrease IGFBP-2 concentrations over the long-term (41) and explain our findings of lower IGFBP-2 concentrations associated with higher animal protein intake in puberty.

Our results indicate a relation between pubertal animal protein intake and GH-IGF-I axis among females only. Most studies in adults or children were conducted in one gender only (12,14,15) and others did not report gender differences (14,33). During puberty, boys have higher testosterone levels than girls, which have been found to increase IGF-I concentrations among healthy men (42). Thus, higher testosterone levels in boys may have overridden a potential effect of animal protein intake on IGF-I. Furthermore, girls have a higher degree of physiological insulin resistance during puberty (43), which may make them more vulnerable than boys to dietary effects on the GH-IGF-I axis. Finally, the smaller variation in animal protein intake levels could partly explain the lack of discernible associations among males.

Our results further indicate that there may be a reversal in the association between animal protein intake and adult IGF-I levels between early life and adolescence. Although no relation was observed with intakes in the period around the adiposity rebound, dietary animal protein intake in early life was inversely associated with IGF-I concentrations in young adulthood among males. This finding is in accordance with the long-term follow-up of a milk intervention (16) and a prospective cohort study (17). In our purely observational study, the association was confined to males. The absence of an association among females may be partly attributable to the small overall sample with consumption data in early life. It has been proposed that higher animal protein intakes in early life may cause an acute increase in hepatic IGF-I production, which then negatively feedbacks to the pituitary GH output, possibly leading to a long-term pro-

FIGURE 2 Relation of dietary animal protein intake in early life (*A*,*B*) (*n* = 68 females, 62 males) and around adiposity rebound (*C*,*D*) (*n* = 94 females, 85 males) to IGF-I in young adulthood among females (*A*,*C*) and males (*B*,*D*) in the DONALD Study, Germany. Data are means (95% CI); model in early life: adjusted for early life (breast feeding) and socioeconomic factors (maternal education and smokers in the household); adiposity rebound: adjusted for age in adulthood, early life (birth weight and length appropriate for gestational age), and socioeconomic factors (maternal education and smoking in the household). Additional consideration of body composition in early life or at adiposity rebound yielded similar results. *P*-trend refers to the *P* value obtained in linear regression models with dietary animal protein as continuous variable. DONALD, Dortmund Nutritional and Anthropometric Longitudinally Designed; IGF-I, insulin-like-growth-factor I; T, tertile.

APPENDIX 4

gramming of the pituitary with lower IGF-I concentrations in adulthood (17).

It remains to be determined whether our data may reflect adaptive responses of the GH-IGF-I axis to intakes of animal protein and/or whether these associations indicate higher or lower risks of future disease. Higher intakes of animal protein during puberty appear to upregulate the entire GH-IGF-I axis, because we observed higher concentrations of both IGF-I and IGFBP-3, but not of the IGF-I:IGFBP-3 ratio (data not shown). In terms of disease risk, a higher concentration of IGF-I has been linked to an increased risk of breast cancer (2), whereas a direct association between IGFBP-3 and breast cancer is currently questioned (3). On the other hand, higher IGF-I concentrations are prospectively related to lower risks of cardiovascular disease (44), osteoporosis (45), and impaired glucose tolerance (46), further complicating a public health appraisal of our results.

A clear strength of our study is its prospective nature, carefully collected repeated dietary data, and the availability of data on several possible confounders. By contrast, the analysis is based on a single measurement of the GH-IGF-I axis in young adulthood to represent long-term circulating levels. However, IGF-I values were reported to have a low intra-individual variation (47). The study sample is relatively small and the DONALD population is characterized by a relatively high socioeconomic status (18). Therefore, extremes of diet or behavior might not be represented in this healthy sample, which is, however, likely to result in an underestimation of the true associations. In addition, the homogeneity of our sample might have reduced our vulnerability to residual confounding.

In conclusion, our data suggest that among females, a habitually higher animal protein intake during puberty may precipitate an upregulation of the GH-IGF-I axis that is discernible in the long-term in young adulthood. By contrast, inverse associations between higher animal protein intakes in early life and IGF-I concentrations among adult males support the idea that habitually higher animal protein intakes in this period may trigger an early programming of the GH-IGF-I axis.

Acknowledgments

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