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The use of metabolomics to improve our understanding of the early programming of diseases

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INHALTSVERZEICHNIS

1 ABKÜRZUNGSVERZEICHNIS

AA	Amino Acids
BCAA	Branched-chain amino acids
BMI	Body Mass Index
CD	Celiac disease
CHOP	Childhood Obesity Project
HLA	Human leukocyte antigen
HP	Higher protein content infant formula
LP	Lower protein content infant formula
MS	Mass spectrometry
NMR	Nuclear magnetic resonance
RCT	Randomized controlled trial
RF	Random forest

2 PUBLIKATIONSLISTE

Diese Dissertation basiert auf folgenden Veröffentlichungen:

Veröffentlichung I:

Investigating the early metabolic fingerprint of celiac disease - a prospective approach. Kirchberg FF, Werkstetter KJ, Uhl O, Auricchio R, Castillejo G, Korponay-Szabo IR, Polanco I, Ribes-Koninckx C, Vriezinga SL, Koletzko B, Mearin ML, Hellmuth C. J Autoimmun. 2016 Aug;72:95-101.

Veröffentlichung II:

Dietary protein intake affects amino acid and acylcarnitine metabolism in infants aged 6 months. Kirchberg FF, Harder U, Weber M, Grote V, Demmelmair H, Peissner W, Rzehak P, Xhonneux A, Carlier C, Ferre N, Escribano J, Verduci E, Socha P, Gruszfeld D, Koletzko B, Hellmuth C; European Childhood Obesity Trial Study Group. J Clin Endocrinol Metab. 2015 Jan;100(1):149-58.

3 SUMMARY

Background:

The hypothesis of early programming states that stimuli applied during the critical or sensitive period can program long-term the structure or function of the organism. Metabolomics is a useful tool to investigate alterations in molecular pathways related to health outcomes. In this doctoral thesis, I used metabolomics data to examine the early programming of two different diseases, namely celiac disease (CD) and obesity.

Methods:

The PreventCD study is a large European study including children at high genetic risk for CD. Metabolomics data was available of 4 months old infants that have not yet been introduced to gluten. I investigated whether there existed a characteristic metabolic profile in infants developing CD during childhood, whether the genotype influenced the metabolic profile, or whether the metabolite species were predictive for the age of CD development. Regarding obesity, I used data from the 6 month visit of the CHOP study which is a randomized controlled European trial. Infants were randomized to a lower or higher protein content infant formula and those of the HP group were found to be at higher risk for obesity at school age. At 6 months of age, metabolomics analyses were performed and I used thus data to examine the programming effect of the higher protein formula milk.

Results:

Regarding CD, evidence was scarce and, based on our results, we could not conclude on a programmed metabolism in 4 months old children developing CD in childhood. Our results furthermore showed that the HLA-genotype had no influence of the infants at this age. As the infants had not been exposed to the triggering factor gluten, we suggested that future studies should target infants after the introduction of gluten. Concerning the comparison of the metabolite profile of the intervention groups of the CHOP study, we could show that the branched-chain amino acids (BCAA) were markedly increased whereas long-chain acylcarnitines were decreased in the infants receiving the HP formula milk. We also found evidence for a saturation of the BCAA catabolism. Thus, the insulinogenic amino acids (AA) and AA promoting IGF-1 secretion might be responsible for the adverse programming of HP children. Furthermore, the decreased beta-oxidation as evidence by decreased acylcarnitine levels in HP children might be linked to the higher leucine levels.

Conclusion:

Metabolomics in epidemiology offers the unique opportunity to perform hypothesis driven mechanistic analyses and detailed results as delivered by metabolomics studies will enable the target development of interventional strategies.

4 ZUSAMMENFASSUNG

Ziele:

Das Konzept von ‚Early Programming' beruht auf der Hypothese, dass bestimmte Stimuli im frühkindlichen Alter langfristige Auswirkungen auf den Organismus haben können. Metabolomics ist eine Wissenschaft, die sich zur Untersuchung von Krankheiten und den damit einhergehenden Veränderungen im Stoffwechsel als sehr hilfreich erwiesen hat. In dieser Doktorarbeit verwendete ich Metabolomics um das Early Programming zweier spezifischen Krankheiten, nämlich Zöliakie und Adipositas, zu beleuchten.

Methoden:

Die PreventCD Studie ist eine europäische Studie, die Kinder mit hohem genetischem Risiko auf Zöliakie einschließt. Im Alter von 4 Monaten, vor der Einführung von Gluten, wurden Metabolomics Messungen bei den Kindern durchgeführt. Anhand dieser Daten konnte ich drei Fragestellungen beantworten: gibt es bereits im Alter von 4 Monaten ein charakteristisches metabolisches Profil in Kindern, die in ihrer Kindheit Zöliakie entwickeln? Hat der HLA-genotyp einen Einfluss auf das Metabolom? Lässt das 4-monats Metabolom Rückschlüsse auf das Alter bei Ausbruch von Zöliakie zu? Im Falle von Adipositas nutzte ich Daten der CHOP Studie, eine europäische randomisierte Studie, in der die Kinder zufällig der Zufuhr einer proteinreichere (HP) oder proteinärmere (LP) Säuglingsmilch zugeordnet wurden. In dieser Studie konnte die Early Protein Hypothese, welche besagt dass hohe Proteinzufuhr im frühkindlichen Alter das Risiko für spätere Adipositas erhöht, bestätigt werden. Im Rahmen dieser Arbeit untersuchte ich den Effekt der Säuglingsnahrung auf den Metabolisms im Alter von 6 Monaten.

Ergebnisse:

Im Falle von Zöliakie konnten wir kein Early Programming feststellen. Der Metabolismus in den 4 Monate alten Kindern ließ keinen Schluss über einen möglichen Zöliakie Ausbruch zu und auch der Genotyp hatte keinen nachweisbaren Effekt auf das Metabolom. Nachdem die Kinder noch kein Gluten zu sich genommen hatten, sollten spätere Studien speziell Kinder nach der Einführung von Gluten betrachten. Was Adipositas betrifft, so zeigte der Gruppenvergleich der randomisierten

Gruppen, dass vor allem die verzweigtkettigen Aminosäuren (AA) sowie deren Abbauprodukte, die kurzkettigen Acylcarnitine, in der HP Gruppe erhöht waren. Des Weiteren fanden wir Hinweise auf eine Sättigung des BCAA Katabolismus. Damit könnten die insulinogenen AA oder AA, welche die IGF-1 Sekretion erhöhen, für den programming Effekt der proteinreicheren Säuglingsmilch verantwortlich sein. Außerdem waren die langkettigen Acylcarnitine in den HP Kindern niedriger konzentriert, was auf eine geringere Fettverbrennung deuten könnte. Möglicherweise könnte Leucin das Bindeglied zwischen den beiden Beobachtungen sein.

Schlussfolgerungen:

Die Anwendung von Metabolomics in der Epidemiologie bietet die einzigartige Möglichkeit um hypothesengesteuerte Studien durchzuführen und Krankheiten auf molekularer Ebene zu untersuchen und zu charakterisieren. Diese detaillierten Ergebnisse werden gezielte und personalisierte Behandlungen ermöglichen.

5 EINLEITUNG

During the last half of the 20th century, more and more evidence accumulated supporting the idea of early programming: It is believed that pre- and perinatal factors influence the risk for adverse adult health outcomes [1, 2]. In the early 60ies, Widdowson and McCance provided first evidence for the long term programming of development and adult body size by postnatal undernourishment in rats [3, 4]. In 1975, Dörner first used the term 'programming' in order to describe the findings on early lifetime factors modulating later health. In his article, he suggested that homeostatic variables, comprising metabolites, hormones, and neurotransmitters, during the critical periods in early life were capable to pre-program brain development, functional disorders, and diseases [5]. Important insights were furthermore added by research on the consequences of the Dutch famine caused by the German blockade of Holland at the end of World War II which used the historical Dutch Famine cohort study including 300,000 19-years old men. These analyses revealed different obesity rates in the offspring according to the exposure of the famine – whether it was during the first or last trimester of their mothers pregnancy [6]. Later, in 1989, Barker et al. brought the idea of the early programming concept back to live. Using data from the Hertfordshire Cohort Study, they found that anthropometric measures at birth were related to later morbidity and mortality [7]. Again three years later, in 1992, Hales and Berker [8] coined the 'thrifty phenotype hypothesis' stating that poor fetal and post-natal nutrition was detrimental to the development and function of organs leading to long-lasting alterations in growth and function of tissues. During the same period, the term 'programming' reappeared in science. This time, however, it referred to the programming of long-term health by nutrition [9]. Since then, based on the results of epidemiological studies, much research has dealt with early life factors and their impact on later health outcomes. The underlying idea is that a stimulus or insult that is applied during the sensitive or critical period can program long-term or lifetime structure and function of the organism [10, 11]. Nowadays, this concept is described as the 'developmental origins of health and disease hypothesis' [1] and the research in this area is promoted by the International Society for Developmental Origins of Health and Disease which was established in 2003 [12].

Early programming furthermore strongly associates with the approaches of personalized medicine. The fact that certain treatment strategies help some patients while they are ineffective for others, urge research to develop personalized treatment strategies and to examine the causes for the ineffectiveness in these patients. The numbers published by the Personalized Medicine Coalition clearly demonstrate the need for individualized medicine: For instance, 43% of the patients having diabetes are not responding to the given treatment. In cancer, this percentage even rises up to

75% [13]. It is believed that different underlying mechanisms leading to the same phenotype are among the reasons for the ineffectiveness of certain treatments in some patients. As in the case of early programming, the challenge lies then in the characterization of these altered metabolic pathways.

The high-throughput omics-sciences (e.g. genomics, transcriptomics, metabolomics) present a powerful tool to dissect the molecular causes and hence the programming of an organism. The study of the dynamics of biological networks will path the way for personalized disease diagnosis and treatment approaches [14]. The neologism 'omics' refers to sciences aiming at a complete quantification and characterization of pools of biological molecules "that translate into the structure, function, and dynamics of an organism or organisms" [15].

Metabolomics

Metabolomics is a very recent addition to the omics-family and it has enjoyed a growing popularity over the last 15 years [16]. It is the science of very small molecules (typically < 1500 Da), the so called *metabolites*, present in a sample, usually cells, tissue, or body fluids. The quantitative complement of all the metabolites is called *metabolome* [17-19]. As of June 2017, there were 74,461 metabolites listed in the Human Metabolome Database [20]. The sample which is used for the determination of metabolites depends on several factors: While the sample of choice should in first instance depend on the research question, the options might be limited as biopsies are not obtainable due to ethical concerns based on the lack of direct benefit for the patient and risks associated with any surgical procedure [21]. Instead, in clinical studies blood or urine are usually sampled as they are considered minimally invasive and this opens up the possibility to perform metabolic phenotyping on large populations [22]. With respect to the measurement concentrations of the metabolites in the sample, there are two different approaches, namely targeted and non-targeted metabolomics. Targeted metabolomics, which is used in this thesis, refers to a method in which a pre-defined set of metabolites is measured; the most common analytical techniques are nuclear magnetic resonance (NMR) and mass spectrometry (MS). Compared to NMR, MS, and especially triple quadrupole MS, yield highly sensitive, robust, and quantitatively reliable measurements of metabolites [23].

The complete metabolite profile obtained is then used for the characterization of a certain physiological state: Metabolites are intermediates or products of the metabolism and "their levels can be regarded as the ultimate response of biological systems to genetic or environmental changes" [18]. Thus, subtle changes in the genome, epigenome, and proteome may be reflected at

the metabolome level and the changes in concentrations may be indicative for pathologic changes before they can be seen on a static histologic slide. Due to the possibility to characterize the metabolic state of a person based on his metabolic profile, metabolomics is a promising tool in biomarker research and personalized medicine [24, 25]. The number of metabolomics research studies for biomarker identification published over the past 10 years has increased exponentially [16]. In 2016, 750 matches on research articles containing the keywords 'metabolomics' and 'biomarker' are found on PubMed. These studies can further be grouped into three categories: While the major group of metabolomics studies is dedicated to the investigation of altered pathways and underlying mechanisms of diseases, somewhat fewer studies are using metabolomics data for diagnostic purposes or focusing on the translation of biomarkers from the lab to the clinic [16].

Outline of this doctoral thesis

In this work, I aim at investigating the early pathogenesis celiac disease (CD) and obesity. Previous studies have outlined the changes in the metabolism coming along with these diseases and there is furthermore evidence for an early programming of the risk for the development of CD and obesity [10, 26-29]. Here, I use metabolomics data in order to investigate in detail if there are early changes in metabolism related to an increased disease risk later in life, and, if so, what pathways are involved. Though the ultimate goal of my research is the same for both diseases, there are methodological differences: While the analysis on the pathogenesis of CD is based on prospective data, the investigations regarding obesity are based on a randomized controlled trial (RCT). Both study designs are suitable for the investigation of early programming effects, but there are subtle though important differences in the interpretation of the results. In the prospective design, there was no focus on one specific programming factor. By looking at the metabolome in relation to a later CD development, we may have detected any early programming factor affecting the metabolism but identification of these factors might have been difficult if not impossible. In contrast, the RCT enables the close examination of one specific factor, in this case high protein intake, and its programming effect of the metabolism. Therefore, a clear characterization of the programming effect and its metabolic consequences is possible. The two studies, research questions, and statistical approaches will be outlined in more detail in the following.

Publication 1 – Early Programming of Celiac Disease

CD is among the most common food intolerances. Its prevalence among the general population is estimated approximately 1% [30]. The gastro-intestinal disorder is characterized by chronic

inflammation of the small intestinal mucosa leading to villous atrophy. CD is triggered by gluten and other prolamines contained in wheat, rye, barley, and other cereals in genetically susceptible individuals [31]. Only adherence to a strict gluten-free diet should reverse the damage to the small intestine and allow for normalization of digestion and nutrient absorption. The PreventCD study has been designed to investigate whether an early introduction of small quantities of gluten were beneficial with regard to reducing the incidence of CD among children from high-risk families. In this RCT, infants were randomized to receive 100 mg of immunologically active gluten daily or placebo from 16 to 24 weeks of age. The study provided evidence that an early exposure to gluten did not alter the risk of CD by 3 years of age. Furthermore, breastfeeding was not found to have positive effects on reducing the risk for CD [32]. This data offers excellent opportunities to examine the early mechanisms of CD. At 4 months of age, we had blood samples from the infants. Using direct infusion mass spectrometry and liquid chromatography-mass spectrometry, we measured amino acids and polar lipid concentrations (acylcarnitines, lysophosphatidylcholines, phosphatidyl-cholines, and sphingomyelins). The longitudinal setup of the study and the long follow-up up to the age of 8 years provided the perfect basis for a prospective examination of the metabolome of these children long before manifestation of the disease. The hypothesis of an altered metabolism from very early age on is based on the results of Bertini et al. [26] and Bernini et al. [27] who provided evidence for an altered plasma metabolic profile in CD patients. CD patients were found to have a different plasma metabolic profile compared to their healthy controls: with respect to malabsorption, to the energy metabolism, and to alterations in gut microbiome and/or intestinal permeability [26]. Bernini et al. then provided evidence that the alterations in the metabolism of CD patients were preceding the development of small intestinal villous atrophy [27]. They compared healthy subjects with those having potential CD (patients with positive CD serology in the absence of any apparent intestinal damage) or overt CD (patients with villous atrophy). The metabolic profile of patients with potential CD was very similar to the one of patients with overt CD. The authors conclude that alterations of the enterocytes and metabolism arise before the observable macroscopic modification of intestinal mucosa [27]. Both research studies were performed on adults. The first publication of this doctoral thesis builds on the findings from Bertini et al. and Bernini et al.: I hypothesize that these changes in metabolism can already be seen in very young infants prior to the introduction of gluten. If this was true, it would mean that the programming of children towards a higher disease risk would take place prior to the age of 4 months, which is the age of the children at blood withdrawal. I aimed at answering three questions: 1) Is there a characteristic metabolic trait in the blood of 4 months old infants developing CD later in life? 2) Does the metabolite profile at 4 months of age contain information

on the age of CD onset? 3) Does the human leukocyte antigen (HLA) genotype influence the metabolism of the children at 4 months of age? Especially research question 2) makes use of the prospective design by analyzing the 'time to event'. Using cox-proportional hazards models, it is possible to quantify the effect of a covariate (in this case the metabolite concentration) on the risk for CD development. In other words, the metabolite concentrations are considered risk factors and their levels in early life are used to predict the child's risk for CD development. However, although the literature suggests that metabolic alterations precede the onset of CD, we could not find any metabolic alterations in 4 months old children developing CD in childhood, preceding the introduction of gluten, nor did we find that the metabolic profile was predictive for the age at CD diagnosis. Also regarding the HLA genotype, we did not find metabolic differences across the genotypes [33].

Publication 2 – The Early Protein Hypothesis on the Programming of Obesity

The second aspect I investigated more closely is the etiology and pathogenesis of obesity by means of metabolomics analysis in an interventional trial. A high body-mass index (BMI) ranges among the top ten largest contributors to global deaths and disability-adjusted life-years among Level 3 [34]. The obesity prevalence rose from 3,2% in 1975 to 10,8% in 2014 in men and from 6.4% to 14.9% in women [35]. This rise furthermore represents a huge economic burden for societies: Obesity was estimated to have a global economic impact of roughly US$ 2.0 trillion – an amount roughly equivalent to the global impact from smoking or armed violence, terrorism, and war [36, 37]. In children, obesity is "one of the most serious public health challenges of the 21st century" [38]. In the 1990s, first evidence arose from epidemiological studies that a higher early protein intake might increase the risk for later obesity: Using data from the French ELANCE longitudinal study, Rolland-Cachera et al. [39] investigated the effect of nutritional intakes at age of 2 years on anthropometric measurements and showed that protein intake was the only nutrient that was associated to body fatness at 8 years of age. This positive relationship was confirmed in several other studies [40-43]. Based on these observations, the 'early protein hypothesis' was postulated [44] and the Childhood Obesity Project (CHOP) was launched [45]. According to the 'early protein hypothesis', an increased protein intake exceeding the infant's requirement, stimulates the secretion of insulin and insulin like growth factor 1 which then enhances early growth and adipogenic activity [46]. The CHOP study was designed to test this hypothesis. In this multicenter European study, infants of parents who decided to formula-feed their child were randomly assigned to receive cow milk–based infant and follow-on formula with lower (1.77 and 2.2 g protein/100 kcal, respectively) or higher (2.9 and 4.4 g protein/100 kcal, respectively) protein

contents for the first year. The primary endpoints of this study were length and weight-for-length z scores at the age of 2 years. While length did not differ between the formula groups, infants of the higher protein (HP) group had an average weight-for-length z score elevated by 0.20 standard deviation compared to the lower protein (LP) group [45]. Also at school age, children of the HP group were at higher risk to be overweight or obese: The risk of becoming obese in the HP group was 2.43 times higher than for children of the LP group [47]. However, it has to be noted that the CHOP study is unique in the sense that it is the only RCT assessing the infant's outcomes after infancy. Therefore, a recent review concluded that there is insufficient evidence on the effect of different protein intakes on the children's growth, body composition, and later risk of overweight and obesity in the long term and that further studies replicating the findings of the CHOP study are needed before certainty on the effects of early protein can be attained [48]. One important step in the investigation of this topic is the examination of the underlying mechanistic pathways. An understanding of these pathways will help to classify the results observed in the clinical trials. In my second publication of this doctoral thesis, I therefore aimed at closing the gap on the mechanistic backgrounds of the effects of the higher protein content in the formula milk. We had blood samples of the infants when they were 6 months of age. We measured plasma amino acid and acylcarnitine concentrations by liquid chromatography coupled to tandem mass spectrometry. Based on this data, I investigated the differences in the metabolism of the infants. I used linear mixed models to test for different mean concentration of the metabolites between the two intervention groups. These models are required to account for the correlation structure arising from the multi-centric study design. I furthermore used random forest (RF) to analyze the differences in the metabolome in the multivariate context. The main finding was on the branched-chain amino acids (BCAA) and their degradation products, the short-chain acylcarnitines, which were highly elevated in the HP group compared to the LP group. We speculate that this is a result of the BCAA escaping the first-pass liver metabolism and that leucine, which is an insulinogenic amino acid, might relate the higher protein intake in infancy with the later increased risk for obesity in childhood. We furthermore found an indication for a saturation of the BCAA degradation capacity which might increase the detrimental programming effect [49].

Conclusion

This doctoral thesis contributes to our understanding of the early programming of CD and obesity and the role that metabolomics plays in this research area. Based on previous findings, I further investigated the pathogenesis of these two diseases at the molecular level. The results add knowledge on the early programming of the diseases and indicate new directions and starting

points for further hypothesis driven research. In the case of CD, my work suggests to focus on older children after gluten was introduced or even later in childhood as alterations might occur only within a shorter period before CD onset. With respect to obesity, I provide evidence that the BCAA might play a pivotal role in the programming of obesity but also with relation to the fatty acid oxidation. Targeted metabolomics, which enables the absolute quantification of known molecules and which was used in this thesis, proved a promising tool in hypothesis driven research. Thus, this work builds a basis for further studies aiming at understanding the mechanisms of the diseases in which targeted metabolomics will offer great possibilities and deliver insights at the molecular level.

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6 VERÖFFENTLICHUNG I

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Investigating the early metabolic fingerprint of celiac disease – a prospective approach

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ABSTRACT

Objectives and study: In the development of Celiac Disease (CD) both genetic and environmental factors play a crucial role. The Human Leukocyte Antigen (*HLA*)-DQ2 and *HLA*-DQ8 loci are strongly related to the disease and are necessary but not sufficient for the development of CD. Therefore, increasing interest lies in examining the mechanisms of CD onset from the early beginning. Differences in serum and urine metabolic profiles between healthy individuals and CD patients have been reported previously. We aimed to investigate if the metabolic pathways were already altered in young, 4 month old infants, preceding the CD diagnosis.
Methods: Serum samples were available for 230 four month old infants of the PreventCD project, a multicenter, randomized, double-blind, dietary intervention study. All children were positive for *HLA*-DQ2 and/or *HLA*-DQ8 and had at least one first-degree relative diagnosed with CD. Amino acids were quantified after derivatization with liquid chromatography – tandem mass spectrometry (MS/MS) and polar lipid concentrations (acylcarnitines, lysophosphatidylcholines, phosphatidylcholines, and sphingomyelins) were determined with direct infusion MS/MS.
We investigated the association of the metabolic profile with (1) the development of CD up to the age of 8 years (yes/no), (2) with HLA-risk groups, (3) with the age at CD diagnosis, using linear mixed models and cox proportional hazards models. Gender, intervention group, and age at blood withdrawal were included as potential confounder.
Results: By the end of 2014, thirty-three out of the 230 children (14%) were diagnosed with CD according to the ESPGHAN criteria. Median age at diagnosis was 3.4 years (IQR, 2.4–5.2). Testing each metabolite for a difference in the mean between healthy and CD children, we (1) could not identify a discriminant analyte or a pattern pointing towards an altered metabolism (Bonferroni corrected $P > 0.05$ for all). Metabolite concentrations (2) did not differ across the HLA-risk groups. When investigating the age at diagnosis using (3) survival models, we found no evidence for an association between the metabolic profile and the risk of a later CD diagnosis.
Conclusion: The metabolic profile at 4 months of age was not predictive for the development of CD up to the age of 8 years. Our results suggest that metabolic pathways reflected in serum are affected only later in life and that the HLA-genotype does not influence the serum metabolic profile in young infants before introduction of solid food.

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1. Introduction

Celiac disease (CD) is a systemic immune-mediated disorder which is triggered by gluten and other prolamins in wheat, barley, rye, and other cereals in genetically susceptible individuals. CD has systemic effects but is mainly characterized by enteropathy with villous atrophy that may result in diarrhea, growth faltering and malnutrition, and other symptoms such as constipation or fatigue [1]. The only known effective treatment is a lifelong, strict gluten-free diet (GFD) [2]. Environmental and genetic factors play an important role in the pathogenesis of CD. Apart from the main environmental trigger gluten, a protein complex formed by gliadins and glutenins [3], other environmental factors such as breastfeeding, time of gluten introduction, or the mode of delivery have been proposed to be associated with the risk for CD. While the influence of some of these environmental factors has been clarified or is still a question of debate [4,5], the relevance of the risk alleles of the Human Leukocyte Antigen (HLA)-DQ2 and HLA-DQ8 for the development of CD is well established: Over 90% of CD patients carry the alleles encoding HLA-DQ2 molecule; most of the remaining CD patients carry the HLA-DQ8 heterodimers. However, although around 25% of the European general population is positive for the HLA-DQ2 heterodimer (and ~50% if HLA-DQ8 is also included), only a small fraction (~1%) will develop CD [6–8]. This indicates that the HLA-DQ2 and/or HLA-DQ8 risk alleles are necessary but not sufficient for the development of the disease [3]. Thus, other genetic and/or environmental factors besides gluten must be involved in the pathogenesis of CD and a closer investigation of the mechanisms involved in the activation and development of the disorder is needed.

Metabolomics, the study of small-molecule metabolite profiles, facilitates the characterization of several pathological conditions such as obesity or cardiovascular diseases [9,10]. The metabolites are intermediates and end products of cellular regulatory processes, and their levels can be regarded to be the result of the interaction of genome, epigenome, transcriptome, proteome, and the environment [11,12]. Investigating the metabolic profile of CD patients is thus a logical consequence. So far, only few studies investigated the differences in metabolic profiles between CD patients and healthy controls [13], and even fewer studies focused on the serum metabolic profile. The results of those studies, however, are quite promising revealing alterations in energy metabolism [14] and suggesting that metabolic alterations may precede the development of small intestinal villous atrophy [15].

Besides this cross-sectional view, it is also of major interest to investigate whether the metabolic profiles of children who will progress to CD later in life differ already at early age. The identification of metabolic markers would represent a significant advance and targets for early interventions and preventive strategies. For instance, studies on type 1 diabetes which shares common alleles with CD, have identified metabolic phenotypes that characterize the early pathogenesis of the disease [16,17]. Using data from the prospective cohort of the PreventCD study, we tested if the metabolic profile of 4 month old children at genetic risk for CD was associated with (1) the development of CD up to school age, (2) the HLA-risk groups, or (3) the age at CD diagnosis.

2. Material and methods

2.1. Study design

The PreventCD project is a prospective, randomized, double-blind, placebo-controlled, dietary-intervention study in children with high risk for CD [5,18]. The first child was included on May 26th, 2007, and the follow-up for this analysis closed on August 26th, 2015. Recruitment of the infants was done consecutively through CD organizations in Croatia, Germany, Hungary, Israel, Italy, the Netherlands, Poland, and Spain. Infants between 0 and 3 months of age were recruited if (i) they had at least one first-degree family member with CD confirmed by small-bowel biopsy, and (ii) were HLA-DQ2 or HLA-DQ8 positive, or otherwise carrying the allele DQB1*02 [18]. Premature infants or infants with syndromes associated with an increased risk of CD, such as trisomy 21 or Turner's syndrome, were excluded. The infants were randomized to the intervention groups and either received gluten (200 mg of vital wheat gluten mixed with 1.8 g of lactose) or placebo (2g of lactose) between 4 and 6 months of age. Randomization took place after blood withdrawal.

The study was approved by the medical ethics committee at each participating center and complied with Good Clinical Practice guidelines. The authors vouch for the veracity and completeness of the data and analyses reported and for the adherence of the study to the protocol.

2.2. Assessment of CD

Children with elevated levels of antibodies indicating CD and/or with clinical suspicion of CD are offered to undergo a small-bowel biopsy to diagnose the disorder. The diagnosis of CD was based on the histologic findings of the small-bowel biopsies, according to the criteria of the European Society for Pediatric Gastroenterology, Hepatology, and Nutrition (ESPGHAN) [19]. The age of the patient at the day of biopsy is considered to be the age at the diagnosis of CD.

2.3. Genotyping

Genotyping for HLA-DQ-alleles was performed by single nucleotide polymorphisms (SNPs) based on the tag-SNP approach (Department of Genetics University Medical Center Groningen, the Netherlands). The HLA-risk groups were defined as follows: (1) DR3-DQ2/DR3-DQ2 (DQ2.5/DQ2.5); DR3-DQ2/DR7-DQ2 (DQ2.5/DQ2.2); (2) DR7- DQ2/DR5-DQ7 DQ2.2/DQ7); (3) DR3-DQ2/DR5-DQ7 (DQ2.5/DQ7); DR3-DQ2/DR4-DQ8 (DQ2.5/DQ8); DR3- DQ2/other (DQ2.5/other); (4) DR7-DQ2/DR7-DQ2 (DQ2.2/DQ2.2); DR7-DQ2/DR4-DQ8 (DQ2.2/DQ8); DR4- DQ8/DR4-DQ8; (DQ8/DQ8)(5) DR7-DQ2/other (DQ2.2/other); DR4-DQ8/DR5-DQ7 (DQ8/DQ7); DR4-DQ8/other (DQ8/other), where other refers to any HLA-DQ haplotype except for DR3-DQ2, DR7-DQ2, DR4-DQ8 or DR5-DQ7 [5,20]:

2.4. Sample collection & quantification of metabolites

Measurements of serum antigliadin antibodies, TG2A, and metabolomics were performed centrally. Blood samples were collected and centrifuged and the frozen serum samples (−20 °C) were sent for determination of CD antibodies to Thermo Fisher Scientific, Freiburg, Germany. The rest of the serum was stored at −20 °C and sent to the central sera bank of the project at the department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Leiden, where it was stored also at −20 °C. Serum samples were available for 230 children who previously have been selected for a study on breastmilk composition during the first months of life and serum composition at 4 month (paper under submission). We took advantage of this collection for the purpose of this study. The sera of these 4 month old infants collected before the start of the dietary intervention were transferred on dry ice to LMU Munich and stored at −80 °C until metabolomic analysis.

Analysis of amino acids (AA) was performed as described previously [21]. Briefly, 10 μl of serum was prepared by derivatization

to amino acid butylester, and determined by ion-pair liquid chromatography (LC). The LC system (Agilent, Waldbronn, Germany) was coupled to a tandem mass spectrometer (MS/MS, QTRAP4000, Sciex, Darmstadt, Germany) with an atmospheric pressure chemical ionization source operating in positive ionization mode. The MS was run in Multiple Reaction Monitoring (MRM) mode.

For quantification of polar lipids, namely free carnitine, acylcarnitines (Carn), lysophosphatidylcholines (LPC), diacylphosphatidylcholines (PCaa), acyl-alkyl-phosphatidylcholines (PCae), and sphingomyelines (SM), serum was diluted with methanol and, after centrifugation, supernatants were injected into the LC-MS/MS system with an electrospray ionization source. The system was used as flow-injection analysis and the MS was run in MRM mode in both positive and negative ionization mode. The structures of the hydrocarbon chains of the polar lipids are indicated by the denotation Cx:y where x and y denote the length of the hydrocarbon chain and the number of unsaturations in the chain, respectively. 'a' indicates that the chain is bound via an ester bond to the backbone, while 'e' indicates binding by an ether bound. 'OH' stands for an additional hydroxyl group and 'DC' for two carboxyl groups in the molecule.

During the quantification processes, the samples of the study centers were randomly distributed across the batches. The entire analytical process was post-processed by the softwares Analyst 1.5.1 and R 3.2.1. To remove the batch effect, we centered the metabolomics data for analysis by subtracting for each metabolite its mean concentration across all batches. Analytes, for which we had measurements for less than 10% of the infants only, were excluded from the analyses. All metabolite concentrations are reported in μmol/L.

2.5. Statistical analyses

In a first step, the data was examined graphically and screened for outliers. We defined a measurement as outlier if its absolute concentration exceeds one standard deviation (SD) away from its neighbor. Baseline characteristics of the children are presented as mean (SD) or count (percentage), for continuous and categorical variables, respectively.

In a first analysis, we investigated if the metabolic profile was associated with the development of CD up to the age of 8 years, coded binary (yes/no). In a second analysis, we examined if the metabolic profile depended on the HLA-risk group. In both analyses, we used linear mixed models (LMM) with random intercept for study center and regressed the metabolite's concentration on the (1) binary CD y/n variable and the (2) categorical HLA-risk group variable. When analyzing the sum parameters, we used the non-batch centered data and calculated LMM with two independent random intercepts for study center and batch number. As the HLA-risk groups sizes were very unequal, we chose the biggest, namely risk group 3, as reference category. ANOVA was used to test for differences between the HLA-risk groups. Possible confounding factors that were considered in both analyses comprised gender, maternal CD status, and age at blood withdrawal. We furthermore applied cox proportional hazards models where we investigated if the metabolite concentration was associated with the time to the CD diagnosis (i.e. the age at diagnosis). In these models, we additionally considered intervention group as confounding factor. Bonferroni correction was applied to all analyses in order to control for false positives. Thus, a *P*-value of below $0.05/183 = 0.00027$ was considered to be statistically significant. Furthermore, as the aforementioned LMM only consider one metabolite at a time, we performed sensitivity analyses using partial least squares discriminant analysis and random forest (RF). RF, an analysis tool especially suitable for high-dimensional setups where the number of variables can be much larger than the sample size, was calculated the same way as described in Kirchberg et al. [22]. To overcome the problem of imbalanced data (few CD diagnoses compared to the number of healthy children), we furthermore downsampled the group of healthy subjects and performed RF on this balanced dataset.

The three analyses were each performed on the whole sample and on a subgroup, defined as follows: Firstly, the infant had to be exclusively breastfed up to the age of 4 months; Secondly, the mother had to be either on a GFD and being CD positive, or not being on a GFD and being healthy (n = 174).

We presented our results graphically in a circular Manhattan Plot summarizing the results of the three approaches. Every metabolite is given a position on the outer circle and three points, each indicating a $-\log_{10}(P)$ value from a model, are positioned on the line connecting the metabolite to the center. Significance can be read by looking at the significance lines at $\alpha = 0.05$ and $\alpha = 0.00027$ (Bonferroni corrected). If a point lies further out than the line, it is significant to the corresponding significance level α.

3. Results

Blood samples were available for 230 children. Baseline characteristics of those infants are listed in Table 1. The mean age at blood withdrawal was 122.3 days (SD, 25.7 d). Up to that day, 184 children (80%) were exclusively breastfed. Among those, 90 mothers were diagnosed with CD and were on a GFD diet, and 84 mothers were healthy and hence not on a GFD diet. The remaining 10 mothers were either on a GFD although being healthy (n = 1), were not on a GFD although having been diagnosed with CD (n = 4), or information on their diet was missing (n = 5). By the end of 2014,

Table 1
Characteristics of the 230 children with metabolite measurements at the age of 4 month. Values are given as n (%), if not indicated otherwise.

	N = 230
Later CD Diagnosis[a]	
No	197 (86%)
Yes	33 (14%)
Female	115 (50%)
Exclusively breastfed up to age 4 month	184 (80%)
Mean age in days at blood withdrawal (SD)	122.3 (25.7)
HLA-risk group[b]	
1	30 (13%)
2	19 (8%)
3	118 (52%)
4	10 (4%)
5	50 (22%)
Mother with CD	
No	116 (50%)
Yes	114 (50%)
Mother on GFD	
No	117 (52%)
Yes	108 (48%)

Abbreviations: CD, celiac disease; GFD, gluten-free diet; HLA, Human Leukocyte Antigen; SD, standard deviation.

[a] Children were aged 6.5 on average when follow-up was closed for this analysis.

[b] Data on the HLA-risk groups were available for 227 children, with HLA typing performed by means of single-nucleotide polymorphisms (SNPs) on the basis of the tag-SNP approach. For the remaining 3 children, the status with regard to HLA-DQ2 and HLA-DQ8 positivity was determined by means of the Eu-Gen Risk test (Eurospital), with no information provided regarding the HLA-risk group. HLA-risk groups were defined as follows: (1) DR3-DQ2/DR3-DQ2 (DQ2.5/DQ2.5); DR3-DQ2/DR7-DQ2 (DQ2.5/DQ2.2); (2) DR7- DQ2/DR5-DQ7 DQ2.2/DQ7); (3) DR3-DQ2/DR5-DQ7 (DQ2.5/DQ7); DR3-DQ2/DR4-DQ8 (DQ2.5/DQ8); DR3- DQ2/other (DQ2.5/other); (4) DR7-DQ2/DR7-DQ2 (DQ2.2/DQ2.2); DR7-DQ2/DR4-DQ8 (DQ2.2/DQ8); DR4- DQ8/DR4-DQ8; (DQ8/DQ8)(5) DR7-DQ2/other (DQ2.2/other); DR4-DQ8/DR5-DQ7 (DQ8/DQ7); DR4-DQ8/other (DQ8/other), where other refers to any HLA-DQ haplotype except for DR3-DQ2, DR7-DQ2, DR4-DQ8 or DR5-DQ7.

and when the children were aged on average 6.5 years (SD, 0.69 yrs), thirty-three out of these 230 children (14%) were diagnosed with CD according to the ESPGHAN criteria. The mean age at diagnosis was 3.8 years (median, 3.4 yrs; IQR, 2.3–5.2 yrs) and youngest age at diagnosis was 1.8 years of age. Of the 174 children who were exclusively breastfed and whose mothers subsisted according to her CD status, 23 (13%) were diagnosed with CD. Children comprised in the subsample were not different to those not comprised with respect to the baseline characteristics listed in Table 1 (data not shown). Regarding the HLA-risk groups, 13% were in the highest risk group. Most children were in the risk group 3 (52%).

3.1. Metabolic profile in relation to a later CD diagnosis and the HLA-risk group

The metabolic profiles of children with and without a later CD diagnosis, or among the five HLA-risk groups, were not different (all corrected $P = 1$; Supplemental Tables 1 and 2). This result did not change in the multivariate LMM where we additionally adjusted for gender, maternal CD status, and age at blood withdrawal. The *P*–values for both analyses are represented graphically in the circular Manhattan-Plot (Fig. 1): Although some metabolites differed at the uncorrected significance level of $\alpha = 0.05$, these differences were far away from significance after correcting for multiple testing. Tables 2 and 3 summarize the concentrations of these metabolites. Regarding the differences between infants with and without development of CD up to school age (Table 2) all of these metabolites are phosphatidylcholines. Among children who were exclusively breastfed up to 4 month of age and whose mothers had a diet consistent to the CD status (thus, were on a GFD and had a CD diagnosis, or were not on a GFD but had a CD diagnosis), this observation becomes even clearer: Some metabolites almost reached the Bonferroni-corrected significance level (Supplemental Fig. 1). For instance, the corrected P-value comparing the concentration of the acyl-alkyl phosphatidylcholine PCae C42:6 between children who will and those who won't develop CD in comparison to healthy infants was 0.08. The calculation of the absolute sum of the phosphatidylcholine species (Fig. 2) revealed that the mean sums were lower in those infants who will develop CD later in life. This difference, however, was not significant at the corrected significance level, neither in the whole nor in the subsample (Supplemental Fig. 2). The (percent) phospholipid composition also was not different (Supplemental Table 3).

The ensemble approach, the RF analysis, also confirmed the non-discriminating nature of the metabolome and dispels the

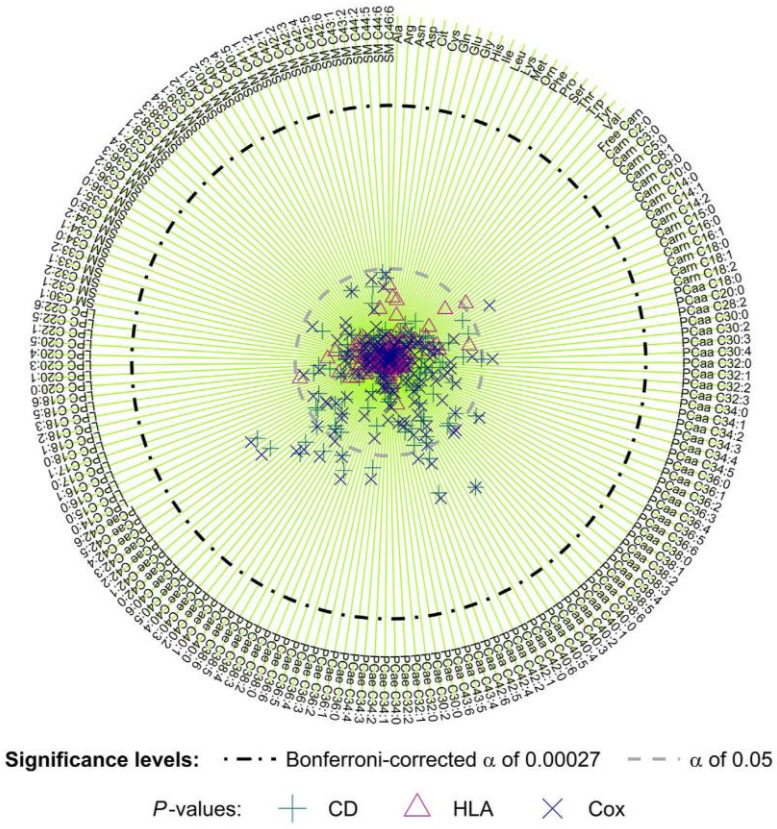

Fig. 1. Circular Manhattan Plot picturing the $-\log_{10}$(*P*-values) of the following three models: (1) linear mixed model (LMM) testing for the difference in the respective serum metabolite between infants that did or did not develop celiac disease ("CD") later in life; (2) LMM testing for the differences in the respective serum metabolite across the HLA-risk groups as defined in Bourgey (2007) ("HLA"); (3) Cox proportional hazards models assessing the effect of the respective metabolite on age at CD diagnosis ("Cox"). LMM were adjusted for gender, maternal CD status, and age at blood withdrawal. Cox proportional hazards models were adjusted for gender and intervention group. Significance is indicated by the grey dashed line ($\alpha = 0.05$), and the black dashed line (Bonferroni-corrected $\alpha = 0.000027$). The center represents an uncorrected *P*-value of 1. Abbreviations: Carn, acylcarnitine; LPC, lysophosphatidylcholine; PCaa, diacyl-phosphatidylcholine; PCae, acyl-alkyl-phosphatidylcholine; SM, sphingomyeline.

Table 2
Mean (SD) of selected serum metabolites of 4 month old infants that did ('CD') or did not ('healthy') develop celiac disease (CD) later in life.

Metabolites (μM)	CD status		Results from LMM				Results from cox proportional hazards model					
			Unadjusted models		Adjusted models[a]		Unadjusted models			Adjusted models[b]		
	Healthy (n = 197)	CD (n = 33)	*P*	Bonferroni-corrected *P*	*P*	Bonferroni-corrected *P*	HR	*P*	Bonferroni-corrected *P*	HR	*P*	Bonferroni-corrected *P*
Diacyl-Phosphatidylcholines												
PCaa C30:4	0.062 (0.02)	0.054 (0.02)	0.048	1	0.052	1	0.98[c]	0.043	1	0.98[c]	0.036	1
PCaa C40:4	4.6 (1.31)	4 (1.11)	0.011	1	0.008	1	0.71	0.013	1	0.69	0.008	1
PCaa C42:5	1 (0.3)	0.89 (0.3)	0.017	1	0.011	1	0.23	0.016	1	0.21	0.01	1
Acyl-Alkyl-Phosphatidylcholines												
PCae C36:0	1.3 (0.4)	1.1 (0.3)	0.0495	1	0.033	1	0.39	0.037	1	0.36	0.023	1
PCae C38:0	4.9 (1.7)	4.2 (1.59)	0.0496	1	0.024	1	0.77	0.028	1	0.75	0.018	1
PCae C38:3	18 (5.12)	16 (4.77)	0.037	1	0.036	1	0.93	0.04	1	0.93	0.034	1
PCae C40:1	3.8 (1.33)	3.3 (1.13)	0.038	1	0.029	1	0.73	0.035	1	0.72	0.024	1
PCae C40:5	9.1 (3.04)	8 (2.73)	0.039	1	0.017	1	0.87	0.032	1	0.86	0.02	1
PCae C40:6	3.5 (1.13)	3.1 (1.15)	0.045	1	0.025	1	0.69	0.023	1	0.67	0.017	1
PCae C42:0	2.2 (1.03)	1.8 (0.8)	0.047	1	0.045	1	0.68	0.055	1	0.67	0.046	1
PCae C42:3	2.9 (0.9)	2.4 (0.8)	0.014	1	0.009	1	0.59	0.011	1	0.58	0.007	1
PCae C42:6	1.8 (0.5)	1.6 (0.4)	0.015	1	0.008	1	0.4	0.011	1	0.37	0.006	1

Abbreviations: CD, celiac disease; HR, hazard ratio; PCaa, diacyl-phosphatidylcholines; PCae, acyl-alkyl-phosphatidylcholines; SD, standard deviation.
[a] Adjusted for gender, maternal CD status, and age at blood withdrawal.
[b] Adjusted for gender and intervention group.
[c] Hazard ratio per nmol/L increase.

Table 3
Mean (SD) of selected serum metabolites of 4 month old infants in the HLA-risk groups. *P*-values to test for differences across the groups were calculated using linear mixed models (LMM).

Metabolites (μM)	HLA-risk groups[a]					*P*-values from LMM			
						Unadjusted models		Adjusted models[b]	
	1 (n = 30)	2 (n = 19)	3 (n = 118)	4 (n = 10)	5 (n = 50)	*P*	Bonferroni-corrected *P*	*P*	Bonferroni-corrected *P*
Carn C8:1	0.094 (0.06)	0.096 (0.06)	0.11 (0.08)	0.16 (0.1)	0.13 (0.09)	0.049	1	0.045	1
LPC C18:3	0.28 (0.1)	0.28 (0.1)	0.3 (0.1)	0.41 (0.3)	0.31 (0.1)	0.042	1	0.06	1

Abbreviations: Carn, acylcarnitine; CD, celiac disease; HLA, Human Leukocyte Antigen; LPC, lysophosphatidylcholine; SD, standard deviation.
[a] HLA typing was performed by means of single-nucleotide polymorphisms (SNPs) on the basis of the tag-SNP approach. HLA-risk groups were defined as in Vriezinga (2014).
[b] Adjusted for gender, maternal CD status, and age at blood withdrawal.

impression from the subsample analysis, this is, different phospholipid levels in healthy and CD children: The out-of-bag error (OOB) rate of 13% (11% in the subsample) equaled in both cases the percentage of CD diagnosis among the studied infants. Thus, RF was not able to classify correctly CD and healthy children but classified everyone as healthy. In the downsampled data, the OOB error was 58%.Table 3 summarizes the results of selected LMM on the HLA-risk groups. The concentrations are similar across the groups (all corrected P-values > 0.05).

3.2. Relating the metabolic profile at 4 month of age with the age at diagnosis

Using cox proportional hazards models, we modelled the predictive power of the metabolite's concentrations on the time to onset of CD. No significant effect was found after Bonferroni correction (Fig. 1, Supplemental Table 1). In fact, significances were similar to those of the LMM which ignores missing data originating from the censoring. Table 2 summarizes the hazard ratios and significances of selected metabolites and underlines this similarity.

4. Discussion

In this investigation on the metabolic preconditions of CD, neither a single metabolite nor any tested combination of metabolites was associated with the CD status at school age, or with the age at CD diagnosis. The HLA-risk group also had no effect on the metabolites' serum concentration.

To our knowledge, there have been no previous studies that examined the associations of early metabolic markers in the blood with a later CD diagnosis. Some publications focus on the metabolic fingerprint for CD in one of the matrices serum, urine, or gut microbiota, but they compare CD patients (potential or overt) with healthy controls [13–15]. Their design differs from ours in that it is cross sectional rather than prospective. Two studies investigated the serum metabolic profile: Bertini et al. [14] found differences in the plasma metabolic profiles of CD patients and healthy controls and defined three components altered in CD: one related to malabsorption, the other to energy metabolism, and a third related to alterations in gut microflora and/or intestinal permeability. Introducing a GFD led to a normalization of the main energy metabolic pathway and a recovery of villous functioning. Bernini et al. [15] put emphasis on the precondition of CD and compared healthy subjects with those having potential or overt CD. Potential CD patients, having the typical immunological features of clear CD in the absence of any apparent intestinal damage, were found to have a virtually similar metabolic profile as the patients with overt CD. They suggest that metabolic and enterocyte alterations of CD arise before the observable modification of jejunal mucosa.

In contrast to these studies, we focused on the alteration of pathways prior to the presence of specific CD antibodies (IgA tissue transglutaminase) or CD onset and, according to the best of our

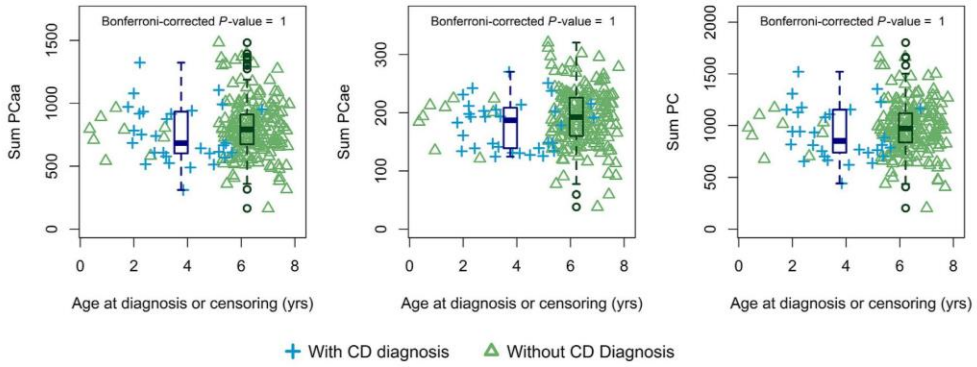

Fig. 2. Scatterplot of the sum of diacyl-phosphatidylcholines (PCaa), acyl-alkyl-phosphatidylcholines (PCae), and all phosphatidylcholines (PC) versus the age at diagnosis (if child was diagnosed celiac disease [CD]; cross) or age at analysis (if child was not diagnosed CD up to that date; triangle). Boxplots show the distribution among the groups. Significance for group difference was assessed with linear mixed models adjusted for gender, maternal CD status, and age at blood withdrawal.

knowledge, we are the first to examine potential metabolic preconditions for CD development in very young infants at genetic risk. Although the results of the cited previous studies point towards alterations in the metabolic profile of CD patients, our findings do not show any such changes in infants aged 4 months prior to dietary gluten introduction. We observed a trend to decreased phospholipid levels in children who later developed CD. However, we strongly emphasize that all corrected *P*-values were above 0.05, no matter which analysis we performed, including those for the sums of the phospholipid species. RF analysis also did not find a discriminative pattern in the metabolites – a fact that underlines the equality of the metabolic profile independently of the sample size. The trend of decreased phospholipid levels in children who later developed CD was stronger in the subsample of mainly exclusively breastfed children. It thus seems that the consumption of liquids or foods other than breastmilk reduces this trend. To exclude a potential influence of a different maternal diet and breastmilk composition of CD mothers, we adjusted the analyses for the maternal CD status and gluten-free diet, but this was also not associated to any metabolite concentration. Decreased phospholipid levels, including phosphatidylcholines and sphingomyelins which both contain fatty acid (FA) chains, have been found in cord blood of children who later progress to type 1 diabetes, a disease which is also associated with the HLA class II genes [17,23]. There are no comparable studies on CD. Studies on the FA composition in CD patients have found ambiguous results: While some found evidence for an altered serum FA composition in adults with CD [24–26], Steel et al. [27] found a similar serum profile of FA in phospholipids in CD and control children. We could not identify any distinct FA species among the phosphatidylcholines (Table 2). Phospholipid composition was also not associated to the CD status at school age. Tjellström et al. [28] who focus on children who already developed CD found a different fecal pattern of some short chain fatty acids (SCFA) between untreated CD children and healthy controls which may reflect bacterial metabolism of undigested carbohydrates secondary to CD-induced malabsorption. This however is not expected to exist at the age of 4 month prior to gluten introduction. SCFA are products of microbial degradation of complex carbohydrates in the colon and are partly transported to peripheral organs for gluconeogenesis, lipogenesis, and other metabolic and immune response pathways [29,30]. It is very likely that this effect would have led to respective alterations in the patterns of esterified serum lipids and acylcarnitines which we did not observe. Therefore, we assume that no major alterations in SCFA are present in 4 month old infants with high risk for CD before gluten introduction. However, further studies measuring serum SCFA are needed to gain certainty on this point.

Regarding the genotype, there were no metabolomic differences across the HLA-risk groups, thus the HLA-DQ genotype does not directly cause the altered metabolism observed later in CD patients. The main genetic predisposing factors are the HLA-DQ alleles accounting for 18% of the genetic risk [31]. Individuals with HLA-DQ2/DQ-8 haplotypes have increased immunological responsiveness to prolamins such as dietary wheat gliadin due to the CD-associated HLA-DQ molecules that bind gluten peptides with a superior ability than the other HLA-molecules [3]. It was found that the HLA-DQ genotype influences the early gut microbial colonization [32,33]. As the metabolome is influenced by the microbiome [34], these findings suggest an altered metabolic profile according to the HLA-risk group which however was not confirmed by this study of infants early before onset of CD. Besides the HLA-genes, also other loci are associated to CD. The total genetic variation is estimated to explain up to 48% of CD heritability [31]. Regulatory mechanisms of CD development may also be modulated by epigenetics and microbiota [6,35].

The strength of this study lies in its prospective character, the long follow-up of up to 8 years, and in the broad assessment of CD related factors, such as information on the mother's diet and CD status as well as the child's HLA-genotype. CD cases were all assessed in an identical way which minimizes the risk of bias. The majority of the infants were exclusively breastfed up to blood withdrawal. Having a sufficient sample size to analyze the children with exclusive breastfeeding and hence to reduce diet induced metabolic variation, we were able to investigate the association of the metabolome not yet influenced by liquids or foods other than breastmilk and future CD development.

5. Conclusions

Our findings do not support a relation of the metabolic profile at age of 4 month prior to gluten-consumption, neither with respect to future CD development nor to the HLA-genotype. Performing metabolomics profiling longitudinally at several time points after dietary gluten introduction might help to better capture the dynamics of the CD development. We conclude that metabolic investigations should focus on children after the introduction of gluten to aid in characterizing and preventing the development of CD.

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Disclosure statement

The authors report no conflict of interest with respect to the study reported.

Clinical trial registration number

PreventCD Current Controlled Trials number, ISRCTN74582487.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jaut.2016.05.006.

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7 VERÖFFENTLICHUNG II

ORIGINAL ARTICLE

Endocrine Research

Dietary Protein Intake Affects Amino Acid and Acylcarnitine Metabolism in Infants Aged 6 Months

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Context: The protective effect of breast-feeding against later obesity may be explained by the lower protein content compared with formula milk. However, the metabolic mechanisms remain unknown.

Objective: We studied the metabolic response to a higher or lower protein supply in infancy.

Design and Setting: The Childhood Obesity Project study is a double-blind, randomized, multicenter intervention trial. Infants were randomized to receive a higher (HP) or lower protein (LP) content infant formula or were breast-fed.

Patients and Interventions: Plasma samples of 691 infants who received formula milk with different protein content (HP, 2.05 g per 100 mL; LP, 1.25 g per 100 mL) or were breast-fed were collected.

Main Outcome Measures: Changes in plasma amino acid and acylcarnitine concentrations of 6-month-old infants according to different dietary protein supply were determined by liquid chromatography coupled to tandem mass spectrometry.

Results: Twenty-nine metabolites differed significantly between the formula groups. Branched-chain amino acids (BCAAs) were the most discriminant metabolites. Their degradation products, the short-chain acylcarnitines C3, C4, and C5, were also significantly elevated in the HP group. A breakpoint analysis confirmed that with increasing BCAAs, the ratio between acylcarnitines and BCAAs decreases. Long-chain acylcarnitines were decreased in HP infants.

Conclusions: BCAAs seem to play a pivotal role in the effect of a high-protein diet on β-oxidation and fat storage. We provide new evidence for a possible saturation of the BCAA degradation pathway that may represent the mechanism by which high-protein intake affects the metabolic regulation. Moreover, it appears to inhibit the initial step of the β-oxidation, thus leading to high early weight gain and body fat deposition. (***J Clin Endocrinol Metab*** **100: 149–158, 2015)**

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Abbreviations: AA, amino acid; AAA, aromatic AA; BCAA, branched-chain amino acid; BCKDH, branched-chain ketoacid dehydrogenase; BF, breast-fed infant; BMI, body mass index; BUN, blood urea nitrogen; HP, higher protein formula group; LOESS, locally weighted scatterplot smoothing; LP, lower protein formula group; OOB, out-of-bag.

There is growing evidence that nutrition and metabolism in pregnancy and early childhood affect health and disease risk in later life (1–3). Breast-feeding, for instance, was found to have a protective effect against obesity in children and adolescents when compared with feeding of conventional infant formula (4–6). It has been suggested that the higher protein supply via conventional formula milk induces elevated insulin and IGF-1 levels (7) and a higher weight gain in infancy which, in turn, is linked to childhood overweight and obesity (8–10). Some studies linked a higher protein intake to elevated insulin and IGF-1 levels (11–13). However, little is known on the metabolic responses to varying dietary protein supply during infancy and the key mediators that might be predictive for later adverse health outcomes.

In previous studies, formula-fed children were found to have increased plasma levels of certain amino acids (AAs), in particular the branched-chain amino acids (BCAAs; isoleucine, leucine, and valine) (13–15) and serum urea nitrogen (16, 17). A cross-sectional study found that BCAA levels were positively associated with the body mass index (BMI) z-score in children aged 8–18 years (18). Also in adults, the circulating levels of BCAAs along with some other AAs were proposed as promising potential biomarkers for developing obesity-associated insulin resistance (19), diabetes (20), and obesity (21). Free carnitine and some long-chain acylcarnitine species were also found to be increased in obese adults (22).

We studied the influence of a different protein supply on plasma metabolic changes in formula-fed infants with a focus on AAs and acylcarnitines, aiming at exploring metabolic mechanisms of early programming of adult health. The evaluated data were collected as part of the European Childhood Obesity Project Trial (23). In this double-blind, randomized clinical trial, it was previously shown that a high protein intake during infancy results in elevated insulin and IGF-1 levels at age 6 months (13), higher weight gain in the first 2 years of life (23), and a higher BMI and an obesity risk at school age (10).

Materials and Methods

Study design

The data evaluated were collected as part of the European Childhood Obesity Project, a double-blind, randomized, multicenter intervention trial conducted in five countries: Germany, Belgium, Italy, Poland, and Spain (24). Briefly, at a mean age of 2 weeks but no later than the age of 8 weeks of life, formula-fed infants were randomly assigned to a higher or lower protein content infant formula (HP and LP groups, respectively). Block randomization was performed with a block size of eight, stratified by sex and study center. Four colors were used for coding the formula groups, two colors per intervention group. To ensure double blinding, the color codes were kept by the manufacturer. The intervention consisted of infant and follow-on formula during the first year of life. Additionally, a nonrandomized group of breast-fed infants (BFs) was included as an observational reference group. Eligible for participation were apparently healthy, singleton, term infants who were born between October 1, 2002, and July 31, 2004. Exclusion criteria for mothers comprised hormonal or metabolic diseases.

Shortly after birth, parents of infants who met the inclusion criteria were invited to participate in a study on the effects of dietary protein on obesity and growth. The recruitment process was designed to encourage breast-feeding. Introduction of other food than study formula or breast milk during the first 3 months after birth was discouraged; afterward the local and family's traditions were respected.

The cow's milk protein content for the LP and HP infant formula was 7.1% and 11.7% of energy (1.25 g protein per 100 mL and 2.05 g protein per 100mL), respectively. Aiming for identical energy content between the LP and HP group, more fat was added to the lower protein formula (3.9 g per 100 mL compared with 3.5 g per 100 mL in the HP group). The intake of carbohydrates (7.5 g per 100 mL) and other nutrients was identical (23). Regarding the total energy content of 69.9 kcal per 100 mL (LP) and 69.8 kcal per 100 mL (HP), they were very similar to the one of human milk, which amounts to 69.7 kcal per 100 mL. The protein and fat content of human milk add up to 1.2 g per 100 mL and 3.6 g per 100 mL, respectively (25). For a detailed description of the contents of the study formulae, see Supplemental Table 1. Follow-on formulae were introduced together with complementary feeding from the fifth month of life onward (protein content 8.8% and 17.6% of energy in the LP and HP, respectively). Regarding AAs, the relative content did not differ between the two formulae with the exception of the LP infant formula that was supplemented with small amounts of arginine and tryptophan to meet legal formula standards (13). The study was approved by the ethics committees of all of the study centers, and written informed parental consent was obtained for each infant (trial registration: ClinicalTrials.gov; identifier: NCT00338689).

Sample collection

Plasma samples were collected at an infant age of 6 months. No blood was collected from children enrolled in Italy. Efforts have been made to draw blood samples 2 or more hours after the last feeding. The plasma samples were stored at −80°C except for Belgium where samples were stored at −20°C.

Amino acids analysis

Eighteen AAs (alanine, arginine, asparagine, aspartic acid, glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, serine, threonine, tryptophan, tyrosine, and valine) were quantified by HPLC with the use of the Pico-Tag method (Waters) as described previously (13). Additionally, citrulline, ornithine, and proline were quantified by ion-pair liquid chromatography coupled to mass spectrometry detection as described previously (26).

Acylcarnitine analysis

For acylcarnitine analysis, 10 μL plasma samples were determined on a hybrid quadrupole mass spectrometer (QTRAP 4000; Sciex) equipped with an Agilent 1200 SL series HPLC

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system (Agilent). Acylcarnitines were qualified and quantified with the Absolute IDQ p 150 kit (Biocrates Life Sciences AG) following the instructions described in the manufacturer's manual. The detailed measurement technique is described in the corresponding US patent US 2007/0004044 (http://www.freepatentsonline.com/y2007/0004044.html). Additionally, as part of the quality control, we calculated the coefficient of variation and excluded the analytes whose coefficient of variation was greater than 20% from the statistical analyses.

A total of 40 acylcarnitines, comprised of acylcarnitines (acylcarnitine Cx:y), hydroxylacylcarnitines (acylcarnitine Cx:y-OH), and dicarboxylacylcarnitines (acylcarnitine Cx:y-DC) as well as free carnitine was determined. Cx:y abbreviates the lipid side-chain composition, whereby x and y denote the number of carbon atoms and double bonds, respectively. OH stands for an additional hydroxyl group in the molecule, and DC indicates that the acylcarnitine has two carboxyl groups.

Unless stated otherwise, we report all metabolite concentrations in micromoles. Additionally to the 62 metabolites measured, we looked at three ratios relating the start substrates of β-oxidation with free carnitine: acylcarnitine C14/free carnitine, acylcarnitine C16/free carnitine, and acylcarnitine C18/free carnitine.

Statistical analyses

All statistical analyses were performed using R (version 3.0.1). First, the data were examined graphically and screened for outliers. An absolute metabolite concentration that lay greater than 3 SD away from its neighbor was considered to be an outlier and excluded from the analysis. Principal component analysis score plots were used as a complementary tool for outlier detection. We computed the mean and the SD of each metabolite in the BF, LP, and HP groups. To test for differences in the concentrations between the groups, we used univariate parametric mixed-effects models to adjust for different study centers.

A Bonferroni correction was applied to counteract the problem of multiple comparisons. P values of less than the adjusted significance level of 7.7×10^{-4} were considered to be statistically significant. The results were represented graphically by plotting the absolute $\log_{10}(P)$ values on the y-axis (referred as Manhattan plots in the following), whereby the direction (below or above 0) was used to indicate in which group the metabolite's concentration was higher. Random forests were used to explore the complex relationship between the formula groups and all metabolites simultaneously.

Variable importance is assessed by permuting the metabolite's values and calculating the out-of-bag (OOB) error. The mean decrease in the OOB error over all trees corresponds to the variable importance. We computed 5000 trees; the number of variables randomly sampled as candidates at each split was set to twice the square root of the total number of metabolites. Variable selection was carried out by successively eliminating 20% of the least important variables. Finally, the solution with the smallest number of metabolites whose OOB error rate was within 1 SE of the minimum error rate of all forests was chosen (1 SE rule).

To explore the relationship between the BCAAs and their respective degradation products, we used local regression [locally weighted scatterplot smoothing (LOESS)] with a second-degree polynomial. For each prediction 75% of the nearest observations were included. Uncertainty of the LOESS curve is displayed in the 95% point-wise confidence interval obtained from 500 bootstrap replications. To allow for the ratio to depend on the respective BCAA concentration, we calculated piecewise linear mixed models with two slopes, including a random intercept for the study center. The breakpoint was estimated using a grid search over the range of the BCAA concentrations. Deviance was computed to express the quality of statistical fitness. Finally, we tested for statistical significance of the breakpoint by comparing the models with and without a breakpoint performing an ANOVA. We applied the same analyses to the blood urea nitrogen (BUN) level to check for an association with the BCAAs that were found to have a breakpoint in the relationship to their respective degradation product.

Results

Blood samples were available for 764 children (86%). Frequencies of blood samples differed with feeding mode

Table 1. Characteristics of the Feeding Groups (LP, lower protein; HP, higher protein; BF, breastfed)

Characteristics	LP (n = 260)	HP (n = 262)	*P* LP vs HP	BF (n = 158)	*P* BF vs LP	*P* BF vs HP
Gender, n, %			.73		.70	.34
Male	130 (50%)	135 (52%)		74 (47%)		
Birth length, cm	50.9 ± 2.75	50.8 ± 2.89	.82	51.4 ± 2.93	.12	.15
Birth weight, kg	3.3 ± 0.32	3.3 ± 0.35	.94	3.4 ± 0.38	.026	.042
Birth BMI, kg/m^2	12.7 ± 1.24	12.7 ± 1.23	.94	12.7 ± 1.18	.26	.23
Age of mother, y	29.0 ± 5.05	29.4 ± 5.22	.44	30.7 ± 4.54	.001	.013
BMI of mother, kg/m^2	23.7 ± 4.41	24.0 ± 4.85	.48	22.5 ± 3.68	.013	.005
BMI of father, kg/m^2	26.2 ± 3.83	26.1 ± 3.72	.87	25.9 ± 3.48	.59	.96
Complications during pregnancy, n, %			.21		.57	.68
Yes	59 (23%)	48 (18%)		38 (24%)		
Smoking during pregnancy, n, %			.07		<.0001	<.0001
Yes	98 (38%)	79 (30%)		12 (8%)		
Birth order, n, %			.67		.74	.18
First child	142 (55%)	140 (53%)		92 (59%)		
second child	89 (34%)	86 (33%)		51 (32%)		
Higher than second child	29 (11%)	36 (14%)		14 (9%)		

Data were expressed as mean ± SD unless stated otherwise.

Table 2. Mean and SD of the Plasma Amino Acid and Carnitine/Acylcarnitine Concentrations in 6-month-old Infants Receiving an Lower Protein (LP) or Higher Protein (HP) Formula or Being Breast Fed (BF)

Metabolite, μM	LP (n = 260)	HP (n = 262)	*P* LP vs HP	BF (n = 158)	*P* BF vs LP	*P* BF vs HP
Essential AAs						
Histidine	106 (26.2)	109 (25.5)	1.00	90 (26.8)	<.0001	<.0001
Lysine	169 (41.9)	201 (59.6)	<.0001	157 (51.8)	.44	<.0001
Methionine	33 (10)	37 (13.5)	<.0001	31 (12.7)	1.00	<.0001
Phenylalanine	74 (18.3)	86 (22.6)	<.0001	62 (21)	<.0001	<.0001
Threonine	130 (38)	148 (44.2)	<.0001	126 (43.7)	1.00	<.0001
Tryptophan	57 (16.1)	68 (19)	<.0001	63 (19.5)	.051	.21
BCAAs						
Isoleucine	66 (21.9)	89 (32.6)	<.0001	63 (24.2)	1.00	<.0001
Leucine	123 (33.9)	168 (54)	<.0001	114 (39.5)	.50	<.0001
Valine	219 (50.1)	308 (84.7)	<.0001	184 (63.1)	<.0001	<.0001
Nonessential amino acids						
Alanine	452 (129)	432 (116)	1.00	433 (107)	1.00	1.00
Arginine	118 (30.7)	110 (29.8)	.19	110 (28.7)	1.00	1.00
Asparagine	55 (13.7)	58 (14.4)	1.00	55 (14.9)	1.00	.24
Aspartic acid	27 (13.3)	29 (15.4)	1.00	29 (16.8)	1.00	1.00
Citrulline	32 (11)	34 (10.3)	.58	27 (11.9)	.007	<.0001
Glutamine	613 (108)	547 (93.7)	<.0001	668 (140)	<.0001	<.0001
Glutamic acid	142 (69.5)	137 (78.8)	1.00	164 (114)	.32	.3
Glycine	277 (77.9)	243 (69.9)	<.0001	230 (63.7)	<.0001	1.00
Ornithine	116 (47.5)	116 (56.2)	1.00	121 (54)	1.00	1.00
Proline	316 (104)	365 (137)	<.0001	319 (134)	1.00	<.0001
Serine	167 (38.8)	164 (37.2)	1.00	186 (44.9)	<.0001	<.0001
Tyrosine	88 (25.1)	104 (34.4)	<.0001	69 (22.7)	<.0001	<.0001
Carnitines/acylcarnitines						
Free carnitine	38 (7.05)	40 (7.32)	<.0001	42 (9.3)	<.0001	1.00
Acylcarnitine C2	5.4 (2.35)	4.8 (2.34)	.14	6.8 (3.04)	<.0001	<.0001
Acylcarnitine C3	313×10^{-3} (0.1)	479×10^{-3} (0.2)	<.0001	449×10^{-3} (0.2)	<.0001	.37
Acylcarnitine C4-OH	72×10^{-3} (0.03)	67×10^{-3} (0.05)	1.00	89×10^{-3} (0.05)	.001	<.0001
Acylcarnitine C3-OH	23×10^{-3} (0.004)	23×10^{-3} (0.004)	1.00	23×10^{-3} (0.004)	1.00	1.00
Acylcarnitine C3:1	6.7×10^{-3} (0.002)	6.5×10^{-3} (0.002)	1.00	6.6×10^{-3} (0.002)	1.00	1.00
Acylcarnitine C4	128×10^{-3} (0.05)	206×10^{-3} (0.09)	<.0001	119×10^{-3} (0.07)	1.00	<.0001
Acylcarnitine C4:1	12×10^{-3} (0.002)	12×10^{-3} (0.002)	1.00	14×10^{-3} (0.003)	<0.0001	<.0001
Acylcarnitine C5	95×10^{-3} (0.04)	154×10^{-3} (0.06)	<.0001	104×10^{-3} (0.05)	1.00	<.0001
Acylcarnitine C5-M-DC	41×10^{-3} (0.006)	39×10^{-3} (0.006)	<.0001	43×10^{-3} (0.007)	.08	<.0001
Acylcarnitine C5-OH	39×10^{-3} (0.009)	45×10^{-3} (0.01)	<.0001	40×10^{-3} (0.009)	1.00	<.0001
Acylcarnitine C5:1	18×10^{-3} (0.007)	21×10^{-3} (0.008)	<.0001	19×10^{-3} (0.007)	1.00	.98
Acylcarnitine C5:1-DC	19×10^{-3} (0.009)	18×10^{-3} (0.01)	1.00	21×10^{-3} (0.007)	.028	<.0001
Acylcarnitine C5-DC	25×10^{-3} (0.008)	20×10^{-3} (0.007)	<.0001	25×10^{-3} (0.009)	1.00	<.0001
Acylcarnitine C6	62×10^{-3} (0.02)	57×10^{-3} (0.02)	.20	73×10^{-3} (0.02)	<.0001	<.0001
Acylcarnitine C6:1	16×10^{-3} (0.003)	14×10^{-3} (0.003)	<.0001	17×10^{-3} (0.004)	.09	<.0001
Acylcarnitine C7-DC	25×10^{-3} (0.007)	22×10^{-3} (0.007)	<.0001	32×10^{-3} (0.01)	<.0001	<.0001
Acylcarnitine C8	148×10^{-3} (0.05)	141×10^{-3} (0.05)	1.00	181×10^{-3} (0.06)	<.0001	<.0001
Acylcarnitine C8:1	168×10^{-3} (0.06)	136×10^{-3} (0.05)	<.0001	167×10^{-3} (0.1)	1.00	<.0001
Acylcarnitine C9	23×10^{-3} (0.02)	23×10^{-3} (0.02)	1.00	31×10^{-3} (0.02)	.001	.001
Acylcarnitine C10	168×10^{-3} (0.06)	149×10^{-3} (0.06)	.007	221×10^{-3} (0.08)	<.0001	<.0001
Acylcarnitine C10:1	177×10^{-3} (0.04)	166×10^{-3} (0.04)	.16	189×10^{-3} (0.05)	.049	<.0001
Acylcarnitine C10:2	33×10^{-3} (0.009)	31×10^{-3} (0.009)	.43	36×10^{-3} (0.01)	.015	<.0001
Acylcarnitine C12	110×10^{-3} (0.03)	96×10^{-3} (0.04)	<.0001	127×10^{-3} (0.04)	<.0001	<.0001
Acylcarnitine C12-DC	49×10^{-3} (0.005)	50×10^{-3} (0.006)	1.00	48×10^{-3} (0.006)	1.00	1.00
Acylcarnitine C12:1	136×10^{-3} (0.03)	133×10^{-3} (0.04)	1.00	163×10^{-3} (0.04)	<.0001	<.0001
Acylcarnitine C14	44×10^{-3} (0.008)	41×10^{-3} (0.01)	.014	57×10^{-3} (0.02)	<.0001	<.0001
Acylcarnitine C14:1	90×10^{-3} (0.03)	88×10^{-3} (0.03)	1.00	118×10^{-3} (0.04)	<.0001	<.0001
Acylcarnitine C14:1-OH	8.4×10^{-3} (0.002)	7.8×10^{-3} (0.003)	.61	13×10^{-3} (0.004)	<.0001	<.0001
Acylcarnitine C14:2	24×10^{-3} (0.01)	$2\,2 \times 10^{-3}$ (0.01)	1.00	28×10^{-3} (0.01)	.027	<.0001
Acylcarnitine C14:2-OH	8.2×10^{-3} (0.002)	8×10^{-3} (0.002)	1.00	9.3×10^{-3} (0.002)	<.0001	<.0001
Acylcarnitine C16	95×10^{-3} (0.02)	87×10^{-3} (0.03)	.004	119×10^{-3} (0.04)	<.0001	<.0001
Acylcarnitine C16-OH	6.7×10^{-3} (0.002)	6.6×10^{-3} (0.002)	1.00	8.1×10^{-3} (0.002)	<.0001	<.0001
Acylcarnitine C16:1	27×10^{-3} (0.007)	26×10^{-3} (0.009)	1.00	35×10^{-3} (0.01)	<.0001	<.0001

(*Continued*)

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Table 2. Continued

Metabolite, μM	LP (n = 260)	HP (n = 262)	*P* LP vs HP	BF (n = 158)	*P* BF vs LP	*P* BF vs HP
Acylcarnitine C16:1-OH	8.6×10^{-3} (0.002)	8.4×10^{-3} (0.002)	1.00	12×10^{-3} (0.003)	<.0001	<.0001
Acylcarnitine C16:2	6.1×10^{-3} (0.002)	5.6×10^{-3} (0.003)	.48	7×10^{-3} (0.002)	.002	<.0001
Acylcarnitine C16:2-OH	11×10^{-3} (0.002)	10×10^{-3} (0.002)	.55	12×10^{-3} (0.002)	<.0001	<.0001
Acylcarnitine C18	31×10^{-3} (0.007)	28×10^{-3} (0.008)	<.0001	57×10^{-3} (0.02)	<.0001	<.0001
Acylcarnitine C18:1	111×10^{-3} (0.03)	101×10^{-3} (0.03)	.008	152×10^{-3} (0.05)	<.0001	<.0001
Acylcarnitine C18:1-OH	8.1×10^{-3} (0.002)	7.6×10^{-3} (0.002)	.46	9.9×10^{-3} (0.002)	<.0001	<.0001
Acylcarnitine C18:2	58×10^{-3} (0.02)	54×10^{-3} (0.02)	.37	57×10^{-3} (0.02)	1.00	1.00

P values were computed using linear mixed models with random intercept for study center. *P* values are adjusted for multiple testing.

(breast-feeders abstained significantly more often) and country (whereas samples were available from almost every child in Poland, 28% of the children in Belgium abstained). Regarding other parameters, we did not find differences between the children with or without available blood samples. Of the 764 infants with available blood samples (BF = 187, LP = 291, HP = 286), 691 (BF = 163, LP = 263, HP = 265) had valid measurements for all metabolites. Eleven infants (BF = 5, LP = 3, HP = 3) were excluded from the analysis due to outliers. The baseline characteristics are shown in Table 1. There were no significant differences between the formula groups, neither in infant characteristics (gender, anthropometry at birth) nor in the parameters regarding pregnancy or parents.

The nonrandomized group of BF infants had a higher birth weight than formula-fed infants. Mothers of BF infants were, on average, slightly older, had a lower BMI, and smoked less often during pregnancy. Plasma concentrations of AAs and acylcarnitines in the LP, HP, and BF groups are summarized in Table 2. Testing each metabolite separately for differences between the LP and HP groups, we found significant differences in 29 of the 62 analyzed metabolites. Figure 1 maps the absolute $\log_{10} P$ values of the comparison of the two formula groups. In a multivariate approach and thus considering the metabolites' dependencies, 14 metabolites (five AAs and nine acylcarnitines) were selected by random forests. Taken together, these 14 metabolites contain most of the information on the difference between the metabolic profiles of infants fed a lower or higher protein formula. Particularly striking are the BCAAs and their degradation products, the acylcarnitines C3, C4, C5, C5-OH, and C5:1, which are significantly higher in the HP group (all adjusted $P < 3.9 \times 10^{-4}$). Random forests selected all of them except for isoleucine. Furthermore, among the 14 metabolites were the essential AA phenylalanine and methionine (higher in HP) as well as the acylcarnitines C5-DC, C6:1, C8:1, C12, and the nonessential AA glutamine (higher in LP). They all were significant to the adjusted significance level of $P < 7.7 \times 10^{-4}$. The respective variable importance values are listed in Supplemental Table 2.

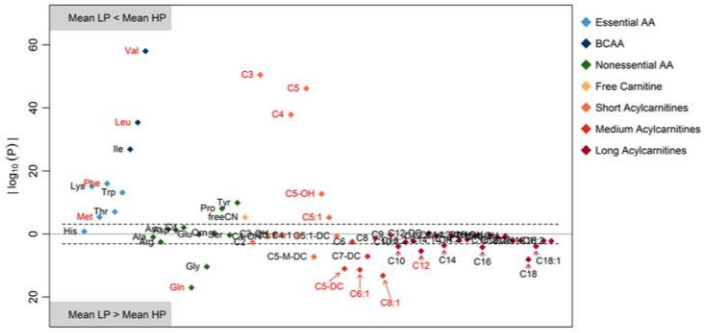

Figure 1. Manhattan plot for the associations of a lower- and higher-protein formula diet with metabolite concentrations. Univariate linear mixed models with random intercept for study center were used. The absolute $\log_{10} P$ values (y-axis) of 62 metabolites are presented, grouped according to their biological function (x-axis). The direction on the y-axis (below or above 0) is used to indicate in which study group the metabolite's concentration was higher. The dashed horizontal lines indicate the adjusted significance level of $\log_{10}(7.7 \times 10^{-4})$. Metabolites depicted by random forests are noted in red.

BCAAs and their degradation products

The relationship of the BCAAs to their respective degradation products is shown in Figure 2. The LOESS curves together with their 95% confidence bands suggest that for substrate-product pairs BCAA/C5 acylcarnitine the concentrations of the degradation product acylcarnitine C5 first increases with the BCAA concentration but then slows down at a certain level. This observation is best seen in the HP infants due to their higher concentrations of BCAAs and acylcarnitines. Regarding the degradation of valine, the plot does not give a clear result: al-

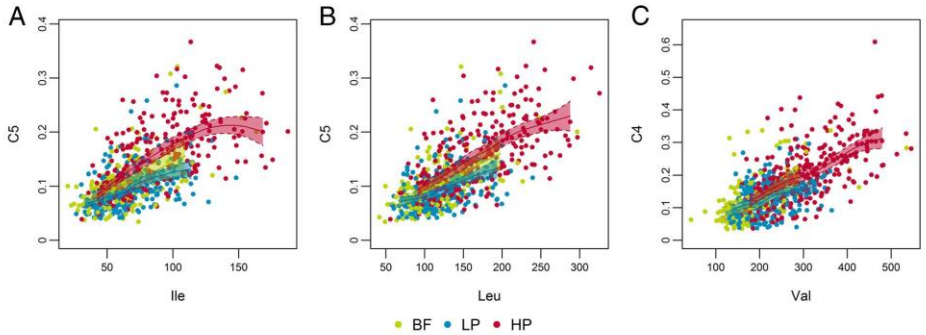

Figure 2. Scatterplot of branched-chain amino acids concentrations vs their respective degradation products by feeding group. The solid line was determined by local regression (LOESS), and the dashed lines represent the 95% confidence intervals obtained by bootstrapping. Concentrations are in micromoles. A, Isoleucine and acylcarnitine C5. B, Leucine and acylcarnitine C5. C, Valine and acylcarnitine C4.

though a slight trend to a stagnating rate is discernible, the confidence band supports a linear relationship. The breakpoint analysis among HP infants statistically confirms the existence of a threshold in the degradation rate for isoleucine and leucine. Models allowing for the degradation rate to depend on the BCAA concentration had a significant better fit than models with a constant degradation rate ($P = 2.3 \times 10^{-4}$ and 0.04 for acylcarnitine C5/isoleucine and acylcarnitine C5/leucine, respectively).

Analyses including all infants irrespective to their feeding group yielded similar results. Figure 3 visualizes the results of the breakpoint models among HP infants showing the predicted population mean. Clearly the relationship of isoleucine and leucine with acylcarnitine C5 changes: although below the breakpoints acylcarnitine levels increase with BCAA levels, they remain constant above the breakpoint. The breakpoints were located at 136 μM (isoleucine) and 234 μM (leucine). Regarding the substrate-product pair acylcarnitine C4/valine, the breakpoint could not be confirmed ($P = .28$). A further analysis focusing on the relationship of BUN (reported in milligrams per deciliter) with isoleucine and leucine yielded the same result

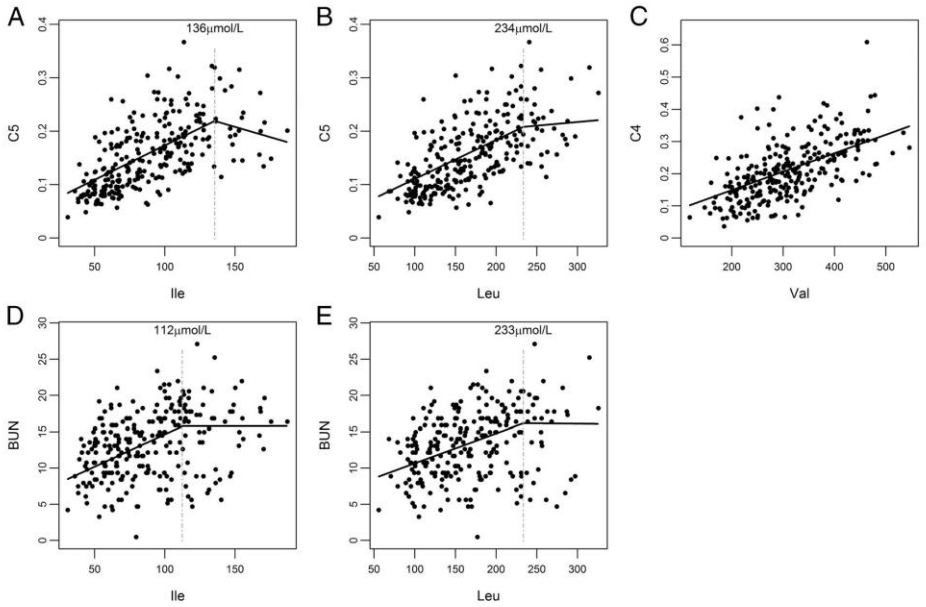

Figure 3. Results of the segmented regression analysis of the BCAA degradation products and BUN among infants on the higher protein diet. Mean acylcarnitine and BUN concentrations averaged over all study centers (black lines) were estimated from piecewise linear mixed models with random intercept for study center. Concentrations are in micromoles (BCAAs and acylcarnitines) and milligrams per deciliter (BUN). A, Isoleucine and acylcarnitine C5. B, Leucine and acylcarnitine C5. C, Valine and acylcarnitine C4. D, Isoleucine and BUN. E, Leucine and BUN.

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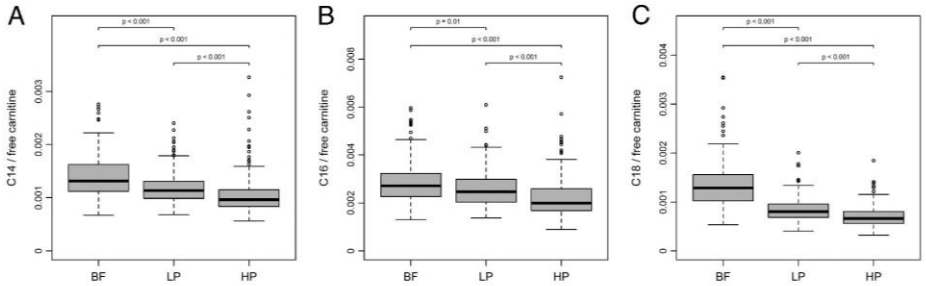

Figure 4. Box plots showing distributions of acylcarnitines C14, C16, C18 to free carnitine ratios across the feeding groups. Significances are computed for all pairwise comparisons using linear mixed models with random intercept for study center. *P* values are adjusted for multiple testing.

(Figure 3). The statistically confirmed breakpoints ($P = 4.5 \times 10^{-4}$ and $P = .04$ for BUN/isoleucine and BUN/leucine, respectively) were similar for isoleucine (112 μM) and leucine (233 μM).

Long-chain acylcarnitines

Among the long-chain acylcarnitines, only C10, C12, C14, C16, C18, and C18:1 were significantly higher in the LP group (adjusted $P < .014$). The ratios relating the starting products of β-oxidation (acylcarnitines C14, C16, and C18) to free carnitine, which itself was significantly higher in children on a higher protein diet (adjusted $P = 3.2 \times 10^{-4}$), were significantly increased in the LP group (adjusted $P = 8.0 \times 10^{-6}$, 6.7×10^{-7}, 7.2×10^{-14} for acylcarnitine C14, C16, and C18, respectively) (Figure 4). The highest ratios, though, were observed in the BF group. They all differed significantly from those of the LP group (adjusted $P < .013$). Regarding the free carnitine levels, they were increased in BF but only significant compared with the LP group (adjusted *P*: LP, 1.5×10^{-4}; HP, 1).

Discussion

Different dietary protein intake had a remarkable influence on the measured metabolites. Even after adjusting for multiple comparisons, 47% of the determined acylcarnitines and AAs differed significantly between the LP and HP groups. Of the 29 significant different metabolites, 14 were selected by random forests.

The increased essential AA concentrations in the HP group as compared with the LP group reflect the higher protein supply via the HP formula because the amino acid composition did not differ between the formulae. Elevated AAs in the plasma of HP-fed children comprise the BCAAs, aromatic AAs (AAAs; phenylalanine, tyrosine, and tryptophan), methionine, lysine, and proline. Several AAs are well known to augment the secretion of insulin, glucagon, or GHs but via different pathways and different potency (27–29). The molecular effect of AAs on GH or IGF-1 levels in humans is, in contrast to animal studies (28, 30), not well described. Different studies showed no effect of orally administered supplements of leucine, glutamine, or arginine on IGF-1 levels in humans (31–33). Dawson-Hughes et al (34) compared the effect of BCAA and AAA supplementation on serum IGF-1 in adults. AAAs induced a higher increase in IGF-1 compared with BCAAs. Hence, higher concentrations of certain essential AAs in the HP group could contribute to an elevated insulin and IGF-1 release in the HP-fed infants, as previously reported for this trial (13). However, special focus is raised on the remarkable elevation of BCAAs in the HP-fed infants. In contrast to other AAs, BCAAs are less metabolized during the first pass in the liver (35, 36). Only a small fraction of the catabolic capacity of the key enzyme of BCAA oxidation, the branched-chain ketoacid dehydrogenase (BCKDH), resides in the liver (35). Thus, the BCAA output of the liver to the blood plasma is higher than for other AAs (37), which explains the highly significant differences in plasma BCAA concentrations between HP and LP.

In the muscle, BCAAs are degraded for energy provision (38), and short-chain acylcarnitines are formed. First, valine, leucine, and isoleucine are transaminased by the branched-chain amino transferase to ketoacids and subsequently degraded by BCKDH to short-chain fatty acids (38). These are bound to carnitine forming the short-chain acylcarnitines C4 and C5 (39, 40). Further degradation results in propionyl-CoA, leading to the acylcarnitines C5-OH and C5:1 as intermediates as well as C3 as end product when bound to carnitine (40).

The slowest step in this degradation and therefore the limiting factor in the BCAA degradation pathway is the degradation step via BCKDH (35). That is, the rate of BCAA degradation depends on the activity of BCKDH. At physiological levels, leucine supplementation increases BCKDH activity (38) to ensure a higher degradation of BCAA in case of higher BCAA availability and to keep

BCAA levels in the physiological range. In our study, with higher plasma concentration of leucine, isoleucine, and valine, the concentration of acylcarnitines C4 and C5 increased until a breakpoint was reached. After this breakpoint, the concentrations of the acylcarnitines C4 and C5 no longer increased with increasing BCAA plasma levels, indicating a saturation of the BCAA degradation pathway. This was especially observed in the HP group because the HP-fed infants had the highest plasma levels of BCAAs. To our knowledge, our study provides the first indication that high-plasma BCAAs may exceed their degradation capacity. On the other hand, the breakpoint may be a result of a stronger efflux of the short-chained acylcarnitines into the mitochondria or of a saturation of the carnitine transfer to the short-chain fatty acids or acyl-CoA. To determine the AA metabolism rate with respect to high BCAA concentrations, we looked at the relation between BUN and BCAAs. This analysis revealed the same nonlinear relation between BUN and BCAAs, confirming the smaller degradation of BCAAs above the breakpoint. The breakpoints of the two analyses, focusing once on BCAAs and short-chain acylcarnitines and once on BCAAs and BUN, were very similar, supporting the hypothesis of a lesser degradation of BCAAs above the breakpoint.

A high BCAA availability exceeding the breakpoint might lead to adverse regulatory effects of BCAAs. This seems especially critical for leucine. Leucine is a potent stimulator of insulin secretion (28). Furthermore, previous studies depicted an influence of leucine on fat metabolism. It was shown that leucine depressed β-oxidation of fatty acids (41, 42). This is in line with our finding that long-chain acylcarnitines ($C \geq 10$) were lower in the HP group than in the LP group. The difference between the concentrations in the two formula groups was largest for the acylcarnitines C10, C12, C14, C16, C18, and C18:1. These acylcarnitines are mainly formed by carnitine palmitoyltransferase 1 at the initial step of the β-oxidation or are the main products of each cycle in the β-oxidation (43). The ratios of the main substrates of carnitine palmitoyltransferase 1 to free carnitine were also reduced in the HP group. This indicates a lower initiation rate of the β-oxidation in the HP compared with the LP group and not an interference with intramitochondrial steps. The reduced β-oxidation initiation may arise through increased leucine levels. Moreover, leucine deprivation resulted in reduced activity of fatty synthase genes (44). Isoleucine and valine deficiency had similar effects (45). This effect of BCAAs on fat metabolism may result in a fat oversupply in human tissues, which may result in lipotoxicity, insulin resistance, and fat storage (43, 46, 47) and build the link between a higher protein intake and a higher BMI (10). Furthermore, high levels of BCAAs affect other amino acid concentrations, including the AAAs. These AAs compete for transport with the BCAAs (48) and possibly are the most potent AAs to promote IGF-1 secretion levels (34). The higher AAA concentrations we observed in HP-fed infants are in line with these mechanisms.

In conclusion, our findings support the positive correlation between high protein intake and early weight gain because insulinogenic AAs and AA-promoting IGF-1 release are elevated in the HP group. We provide new evidence for a possible saturation of the BCAA degradation pathway at a certain level, which may constitute the mechanism by which a high-protein diet affects metabolic regulation. This hypothesis of a lower degradation rate at elevated BCAA levels was further supported by the likewise nonlinear relation between BUN and BCAAs. Furthermore, the lower β-oxidation initiation rate, which was found in infants on a higher-protein diet and likely results in elevated fat storage and increased weight gain in infants, may be a consequence of high leucine levels. Considering the insulinogenic effect of BCAAs and the positive effect on AAA concentrations, which seem to be the most potent promoter of IGF-1 secretion, our results provide an explanation for the early protein hypothesis at the molecular level. Based on these results, we encourage further investigation of the capacity limitation of the BCAA degradation pathway and the avoidance of dietary choices in infants that induce high plasma concentrations of BCAAs.

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