Good, Bad, or Risky?

Intestinal permeability to protein: Human and *in vitro* studies

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Chapter 1

General Introduction

> ## Thesis aim:
> To characterise intestinal permeability to dietary proteins and peptides *in vivo* and *in vitro*.

Background

This general introduction will elaborate on the physiological characteristics of intestinal integrity and permeability. Specific attention will be paid to the digestion and absorption of protein. Subsequently, the two approaches to study intestinal integrity as applied in this thesis, an *in vivo* human intervention model and an *in vitro* human cell model, will be introduced. Finally, the aims and outline of this thesis will be given.

In advance, I would like to emphasize the importance of the gut in human wellbeing; this organ plays a pivotal role in the maintenance of homeostasis and health. Many physiological and pathophysiological conditions can cause dysregulation of the integrity of the epithelial barrier. Examples are autoimmune diseases (T1D, MS, etc.) [1], food allergies [2], diseases related to the gut (Celiac Disease, Crohn's Disease, etc.) [3, 4], and diseases related to chronic inflammation (IBD, chronic fatigue syndrome, etc.) [5, 6], but also physiological conditions such as strenuous physical exercise [7, 8], psychological stress [9] and the use of alcohol or specific medication (non-steroid anti-inflammatory drugs, NSAIDs) [10-12] have been implicated in dysregulation of intestinal integrity. In order to find potential mitigation strategies to support the integrity of the intestinal barrier, it is important to define intestinal barrier integrity and to determine possible mechanisms underlying its dysregulation.

The gut serves two major functions to support whole body homeostasis and health. First it is the site for the digestion of food and subsequent absorption of water, minerals, and nutrients and second it protects the body's internal environment from potentially harmful compounds and micro-organisms in the external environment. Therefore, the small intestine has to form a selective semi-permeable barrier with a high capacity of digestion, absorption and metabolism of essential dietary nutrients, while, at the same time, it has to prevent the invasion of potentially harmful dietary and microbial antigens and commensal and pathogenic bacteria and toxins. When this epithelial barrier functions in a proper manner, the integrity of the intestinal barrier is high. Dysregulation of this integrity of the epithelial barrier can lead to an increase in intestinal permeability and translocation of potentially harmful compounds, which contributes to local and systemic immune activation [13].

Intestinal integrity

Intestinal integrity refers to the two key features essential for maintaining intestinal barrier function. These are, first, the physical and biochemical epithelial barrier, and second the intestinal immune system. The physical and biochemical barrier of the intestine consists of a polarized monolayer of intestinal epithelial cells, also named enterocytes, covered by a continuous mucus layer. These enterocytes are rapidly renewed, with high rates of differentiation and shedding. The enterocytes are held together by three different interconnected protein networks: desmosomes, adherens junctions, and tight junctions (**Figure 1.1**). Together, these networks are called junctional complexes [14]. These junctional complexes interact with neighbouring cells, and are therefore of importance to facilitate intercellular communication. They are furthermore connected with the cytoskeleton and are thereby also of importance to preserve the epithelial architecture and to facilitate cell migration during epithelial cell turnover [15, 16].
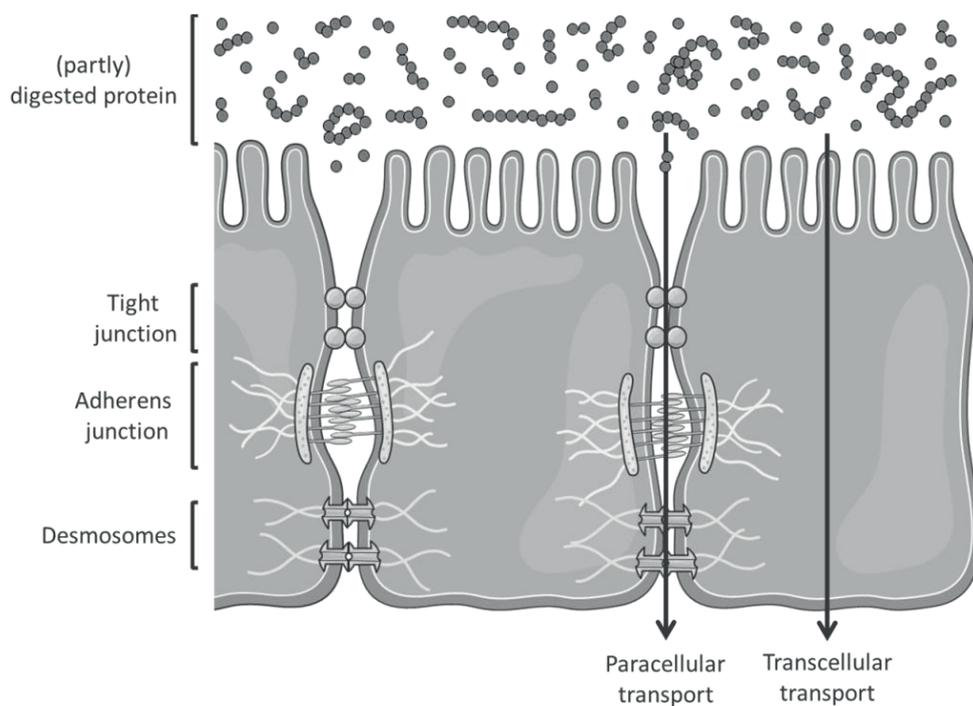
Figure 1.1. Intercellular junctions between and transport routes across intestinal epithelial cells
The intercellular space between intestinal epithelial cells is sealed by different protein complexes, including tight junctions, adherens junctions, and desmosomes. The adherence junctions and desmosomes provide strong adhesive bonds between the epithelial cells and support intercellular communication, but they do not determine paracellular permeability. The tight junctions consist of transmembrane proteins and intracellular proteins connected to the actin cytoskeleton, creating a selective barrier for the paracellular transport pathway. This figure was produced using Servier Medical Art. (http://www.servier.com).

Nutrients and other compounds can cross the epithelial barrier via transcellular and paracellular routes (**Figure 1.1**). The transcellular route (through the enterocyte) is mainly used to absorb water and nutrients [17, 18]. The paracellular route (in between the enterocyte) is facilitated by the paracellular space surrounding the brush border surface of the enterocytes. The size of this paracellular space is particularly determined by the before mentioned junction complexes [14]. These complexes are important regulators of the paracellular pathway and allow the passage of water, solutes, and ions, but under normal conditions prevent the absorption of larger nutrients, such as peptides and protein-sized molecules[19, 20]. The tight junctions limit the flux of solutes and hence are considered the rate-limiting step in the paracellular route of transport [21]. The tight junctions are highly dynamic complexes, continuously monitored and regulated by signalling molecules [22]. Many different protein families form these highly dynamic complexes which anchor two neighbouring enterocytes, such as zonula occludens, occludins, claudins, and junctional adhesion molecules. Each of these protein families has a specific function with regard to the regulation of intestinal permeability. The claudin family is the main determinant of regulation of paracellular permeability by the tight junctions [23]. Some of these claudins play a role in decreasing the paracellular permeability by tightening the junctions, examples are claudin 1, 4, 5, and 8 [24-27]. In contrast, claudin 2, 7, and 12 have been described to increase paracellular permeability [28-30], for example by forming water pores accessible for cations [28]. The structure and function of the different tight junction proteins have been extensively reviewed by Suzuki et al. [21].

Digestion and absorption of dietary protein

Characteristics of the gastrointestinal tract for the digestion and absorption of dietary proteins have been described since the late 1970s, but detailing these processes is ongoing [31].

Digestion of dietary proteins starts in the stomach under the influence of gastric acid and pepsinogens secreted by the chief cells of the gastric gland. Gastric acid not only aids to denature protein, it also lowers the pH. This converts the inactive pepsinogen into the active pepsin. Proteins denatured by the low pH alter their conformational structure, which makes their peptide bonds readily accessible for pepsin. Pepsin cleaves the dietary protein at specific points of the peptide chains, preferably between hydrophobic and aromatic amino acid residues, into polypeptides. Once the now partially hydrolysed protein enters the small intestine, digestion is taken over by proteases excreted by the pancreas. Trypsin, chymotrypsin, elastase, and carboxypeptidases cleave the polypeptides into oligopeptides, tripeptides, dipeptides, and single amino acids. Each of these proteases has its own specificity. Endopeptidases cleave the peptide chains at the peptide bonds of specific amino acids, while exopeptidases cleave specific amino acids one at the time from the carboxyl end of the peptide chains. Additionally, the brush border contains aminopeptidases which further cleave amino acids from the amino end of the peptide chains. Some proteins or

protein-derived peptides are relatively resistant to digestion due to their structural features. For example, proteins that contain many disulphide bonds are resistant to denaturation by the gastric low pH [32]. Without this denaturation step, cleavage sites for the digestive enzymes are much less accessible for cleavage. Next to this, due to the different specificities of each of the digestive enzymes, some proteins and peptides are more difficult to cleave than others and this is especially true when they are not denatured. Examples of proteins and peptides where their bioactivity and immunogenicity depend on their partial resistance to digestion are food allergens and bioactive peptides [33]. The bioactive peptides resulting from partial protein digestion have amino acid sequences relatively resistant to the action of the digestive enzymes; the sequences contain for example several proline residues [34]. For that reason they can be absorbed intactly and execute their bioactive properties systemically, which may, for example, be anti-hypertensive effects. The bioactivity of a large number of peptides has been described for partially digested proteins, such as milk proteins [35].

After the dietary proteins have been broken down by digestive enzymes to oligopeptides, tri- and dipeptides and free amino acids, these have to be absorbed by the enterocytes. Of the two main routes for the absorption of nutrients across the epithelial barrier (**Figure 1.1**), the transcellular route through the epithelial cells is a collection of three different mechanisms of transport: (1) passive diffusion, (2) active and passive, carrier-mediated transport, and (3) endocytosis and exocytosis [36]. The first does not seems to play a role in the absorption of peptides and amino acids, but there are many different amino acid transporters [31] with affinities for different types of amino acids and with different types of active and passive transport. The di/tripeptide transporter (PEPT1) is the major transporter accountable for the absorption of oligopeptides across the enterocytes. Endocytosis and exocytosis play a role in the absorption of peptides and possibly even intact proteins [37]. Immediately after birth, relative large quantities of larger peptides are absorbed across the intestinal epithelial barrier. This absorption of larger peptides or intact protein transfers passive immunity from mother to child and is suggested to discontinue several months after birth after closure of the gut [38, 39].

The question whether larger peptides or intact proteins are absorbed also in adult healthy life is an intriguing one. Gardner [40, 41] already discussed upon this topic, but there is still limited evidence that larger peptides and protein are taken up intactly by the intestine [42, 43]. From the existence of food allergies, however, it can be deduced that indeed a small amount of immunologically active food protein can be taken up and enter the circulation [44]. In this thesis, I will examine whether this also applies for several specifically chosen dietary proteins and peptides and whether a physiological challenge, in this case exercise, can affect this uptake.

Intestinal permeability- the definition

I would like to use this paragraph to clearly define the expression 'intestinal permeability' as it will be applied throughout this thesis. Definitions in literature are not always clear and often intestinal permeability refers only to the ability of compounds to cross the intestinal epithelial barrier via the paracellular route [45]. The focus in this thesis will be on intact peptides and proteins, which are, under normal conditions, crossing the intestinal epithelial barrier only to a very minor extent. I would like to simplify the definition of intestinal permeability to: 'the ability of compounds to cross the intestinal epithelial barrier', independent of the route involved (**Figure 1.2**).
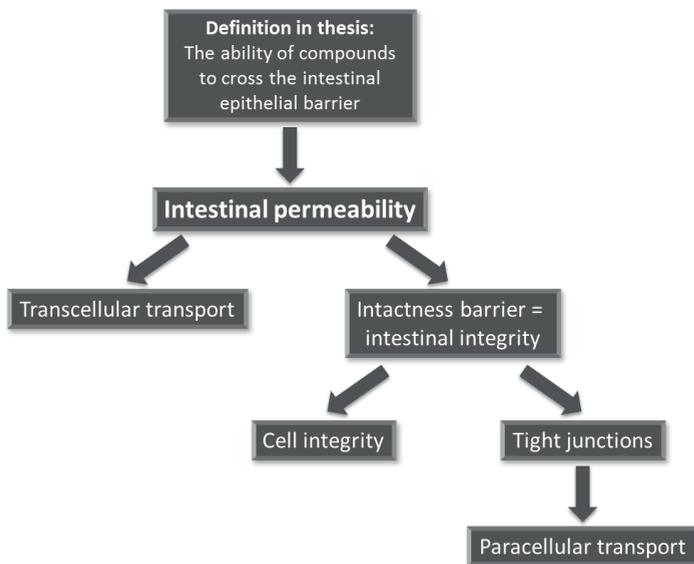
Figure 1.2. Schematic overview of the definition of intestinal permeability.
This scheme explains the definition of intestinal permeability as applied in this thesis and shows the different factors, transcellular transport and intestinal integrity, which can influence intestinal permeability. Intestinal integrity is determined by the cell integrity, which can decrease with the loss of cells, and the tight junctions, which determine paracellular transport.

The main reason for this simplification is that in the studies performed within this thesis, the cause for changes in the amount of a specific dietary protein that crosses the intestinal epithelial barrier is not directly determined. There are several ways in which dietary proteins can cross the intestinal epithelial barrier to a greater extent when this barrier is physiologically challenged. First, there could be increased transcellular transport and second, compromised intestinal integrity could lead to increased leakage of the dietary proteins. The latter could be due to epithelial cell loss and thus changes in cell integrity or due to changes in the tight junctions which regulate paracellular transport. Because I cannot directly

distinguish between these different causes in the *in vivo* studies, it is important to take established markers for each of these factors into account.

Intestinal permeability *in vivo*

Specific inert markers of different sizes are used to determine the ability of compounds to cross the epithelial barrier via the paracellular and transcellular route. The sugar test is most frequently used in the *in vivo* human situation [46]. This test has already been developed and applied from the 1970s [47, 48], mainly to assess the leaky gut in intestine-related diseases. Sugar tests consist of one or more inert sugars. When the dual sugar test is applied, this contains one monosaccharide suggested to freely move across the small intestine via the transcellular pathway, such as mannitol or rhamnose, and one disaccharide suggested to be able to cross the intestinal epithelial barrier only via the paracellular pathway, such as the disaccharide lactulose [48, 49]. The use of two sugars instead of just one is suggested to correct for potential inter-individual differences such as in intestinal transit and renal clearance [48], although only using disaccharide-to-monosaccharide ratios could also lead to wrong conclusions and underestimation of intestinal permeability [50]. More recently, multi-sugar assays containing 3-5 different inert sugars have been developed to take into account gastric and/or colon permeability, in addition to small intestinal permeability [51, 52], or to test for more different types of intestinal transport [53].

Exercise and intestinal permeability

In this thesis, the main focus will be on the effects of a physiological challenge, in this case exercise, to induce intestinal permeability to dietary proteins and peptides. The processes involved in intestinal digestion, absorption and metabolism mainly take place during rest. During exercise, there is a shift in the blood flow from the gastrointestinal tract towards the muscle, which is in high need of nutrients and oxygen under exercise circumstances [54, 55]. Because blood flow is redistributed towards active muscles and lungs, less oxygen and energy substrates are available for the gastrointestinal tract. This has been reported to lead to various gastrointestinal complaints. The effects resulting from the reduced splanchnic blood flow have been shown to lead to exercise-induced intestinal permeability, which has been studied in several human intervention studies [7, 55-65].

Intestinal permeability *in vitro*

Many different *in vivo* and *in vitro* methods have been applied to study the small intestinal epithelial barrier function in humans, such as animal models, everted gut sac, isolated intestinal segments, Ussing chambers, and cultured cell lines. Most of these are based on animals or animal-derived materials. Within this thesis I have chosen to use a human cell line

culture, next to the human intervention studies. *In vivo* models are the best representation of the actual complex physiological situation, but *in vitro* models enable the study of specific cellular mechanisms and can be developed into relatively cheap and quick models that can be used for example for screening purposes. There are different human cell lines that are used to model the small intestinal epithelium. The most well-known cell lines are HT-29, T84, and Caco-2 cells. Caco-2 cells are the most frequently used cellular models to study intestinal epithelial permeability [66, 67]. Caco-2 cells can spontaneously differentiate into polarised intestinal cells in culture [68, 69]. Even though they are derived from a colon adenocarcinoma, upon differentiation they show many characteristics of small intestinal enterocytes [68, 69]. They have the ability to differentiate into a polarized monolayer on microporous filters after which they form domes, and present tight junctions and an apical brush border with its associated hydrolases, such as sucrase-isomaltase. In that respect, the Caco-2 cells gain favour over the other human colon carcinoma cell lines to study small intestinal digestion and absorption. The main advantage of HT-29 cells, however, is that this cell line has goblet cell phenotypic clones that are able to excrete mucus upon differentiation [69, 70]. Therefore, co-cultures between Caco-2 cells and HT-29 cells have also been applied, mainly in the field of drug transport [71]. Caco-2 cell monolayers provide different routes of transcellular transport as well as paracellular transport and thus represent the two main routes of nutrient transport [72].

In this thesis, I questioned the importance of mitochondrial oxidative metabolism for maintenance of intestinal integrity using differentiated Caco-2 cell monolayers. Mitochondrial dysfunction has been associated with intestine-related diseases such as inflammatory bowel disease [73], in which intestinal permeability is one of the key features. Caco-2 cells, however, are usually cultured in high glucose culture media. This culture condition, unlike the *in vivo* situation, facilitates survival of Caco-2 cells on glycolytic metabolism and hampers the study of the potential role of the mitochondria in intestinal permeability. This is due to the so-called 'crabtree' effect, which is defined as an inhibition of mitochondrial oxidative phosphorylation due to enhanced glycolysis [74]. Even though the mitochondria are functionally completely intact, stimulation of these cells with mitochondrial inhibitors has little effect on their energy status [75]. Cells can be cultured in glucose-depleted media to overcome this effect. The replacement of glucose with galactose has been shown to enhance mitochondrial oxidative phosphorylation in different cell types [75-78]. In this thesis, I tested if this also holds true for Caco-2 cells.

Intestinal permeability of the polarized Caco-2 monolayers can be evaluated by measuring transepithelial resistance and/or the assessment of fluxes of marker molecules such as mannitol, dextrans, and inulin [21, 49, 79, 80]. The principal of the fluxes of marker molecules is similar to the sugar test *in vivo*. The transepithelial resistance reflects the ion flux through the paracellular space and is less determined by the size of the paracellular space [81].

Aims and outline of this thesis

In view of increased use of novel food sources and the prevalence of food allergies, it is important to understand how intestinal permeability to dietary proteins and peptides can be affected by normal physiological challenges, such as exercise, and to understand the underlying mechanisms involved.

Therefore, the overall **aim of this thesis** was to characterise intestinal permeability to dietary proteins and peptides *in vivo* and *in vitro*. To this end, two different exercise interventions are evaluated, with two different populations and two different protein sources. Next, an *in vitro* intestinal epithelial Caco-2 cell model was developed to study the role of mitochondrial oxidative metabolism in maintenance of intestinal integrity.

Chapter 2 describes the first of those exercise interventions, which was incorporated in the Protégé study. In this study, I compared the effect of the exercise intervention on the intestinal paracellular permeability by comparing the passage of lactulose with that of casein, as measured by the urinary accumulation of a casein-derived peptide, betacasomorphin-7. Within this study, many stress-related parameters of exercise were analysed as well. As it was chosen to design the Protégé study in such a way that well-trained healthy male cyclists completed the exercise intervention not only once, but twice with one week wash out in between, the test-retest repeatability of exercise-induced effects could also be assessed. This part of the Protégé study is described in **chapter 3**. For the next two studies, PEANUTS Pilot and PEANUTS Study, it was the aim to evaluate a less strenuous exercise intervention with also less trained, but overall healthy men and women. The focus now switched to intestinal permeability towards a dietary protein from peanuts, Ara h 6. **Chapter 4** focusses on the development of the method within the PEANUTS Pilot study to detect this peanut-derived protein. Several factors that could potentially hamper the detection of Ara h 6 in serum after peanut consumption were evaluated. In **chapter 5**, this knowledge was taken into practice with the PEANUTS Study in which the developed method was applied to compare the effect of the exercise intervention on the paracellular permeability to lactulose with the passage of the peanut-derived protein Ara h 6. In **chapter 6**, the focus moved from *in vivo* human trials to *in vitro* mechanistic studies. A Caco-2 intestinal epithelial cell model was developed to evaluate the potential role of the mitochondrial oxidative metabolism in intestinal integrity. This part was performed to gain more understanding in one of the potential mechanisms that could underlie the exercise-induced increase in intestinal permeability. Last, **chapter 7** discusses the main findings of this thesis, strength and weaknesses of the study designs, future perspectives and closes with some general conclusions. This general discussion is followed by a brief summary in English of the main issues addressed in this thesis.

References

1. Fasano, A., Leaky gut and autoimmune diseases. Clinical reviews in allergy & immunology, 2012. 42(1): p. 71-78.
2. Perrier, C. and B. Corthesy, Gut permeability and food allergies. Clinical & Experimental Allergy, 2011. 41(1): p. 20-28.
3. Irvine, E.J. and J.K. Marshall, Increased intestinal permeability precedes the onset of Crohn's disease in a subject with familial risk. Gastroenterology, 2000. 119(6): p. 1740-1744.
4. Fasano, A., et al., Zonulin, a newly discovered modulator of intestinal permeability, and its expression in coeliac disease. Lancet, 2000. 355(9214): p. 1518-1519.
5. Xavier, R. and D. Podolsky, Unravelling the pathogenesis of inflammatory bowel disease. Nature, 2007. 448(7152): p. 427-434.
6. Maes, M. and J.-C. Leunis, Normalization of leaky gut in chronic fatigue syndrome (CFS) is accompanied by a clinical improvement: effects of age, duration of illness and the translocation of LPS from gram-negative bacteria. Neuroendocrinology Letters, 2008. 29(6): p. 902.
7. Pals, K.L., et al., Effect of running intensity on intestinal permeability. Journal of Applied Physiology, 1997. 82(2): p. 571-576.
8. de Oliveira, E.P. and R.C. Burini, The impact of physical exercise on the gastrointestinal tract. Current Opinion in Clinical Nutrition & Metabolic Care, 2009. 12(5): p. 533-538.
9. Kelly, J.R., et al., Breaking down the barriers: the gut microbiome, intestinal permeability and stress-related psychiatric disorders. Frontiers in Cellular Neuroscience, 2015. 9.
10. Bjarnason, I., K. Ward, and T. Peters, The leaky gut of alcoholism: possible route of entry for toxic compounds. Lancet, 1984. 323(8370): p. 179-182.
11. Sigthorsson, G., et al., Intestinal permeability and inflammation in patients on NSAIDs. Gut, 1998. 43(4): p. 506-511.
12. Bjarnason, I. and K. Takeuchi, Intestinal permeability in the pathogenesis of NSAID-induced enteropathy. Journal of Gastroenterology, 2009. 44(19): p. 23-29.
13. Farhadi, A., et al., Intestinal barrier: an interface between health and disease. Journal of Gastroenterology and Hepatology, 2003. 18(5): p. 479-497.
14. Turner, J.R., Intestinal mucosal barrier function in health and disease. Nature Reviews Immunology, 2009. 9(11): p. 799-809.
15. Ey, B., et al., TLR2 mediates gap junctional intercellular communication through connexin-43 in intestinal epithelial barrier injury. Journal of Biological Chemistry, 2009. 284(33): p. 22332-22343.
16. Harris, T.J. and U. Tepass, Adherens junctions: from molecules to morphogenesis. Nature Reviews Molecular Cell Biology, 2010. 11(7): p. 502-514.
17. Daugherty, A.L. and R.J. Mrsny, Transcellular uptake mechanisms of the intestinal epithelial barrier Part one. Pharmaceutical Science & Technology Today, 1999. 2(4): p. 144-151.
18. Laforenza, U., Water channel proteins in the gastrointestinal tract. Molecular Aspects of Medicine, 2012. 33(5–6): p. 642-650.
19. Baum, B. and M. Georgiou, Dynamics of adherens junctions in epithelial establishment, maintenance, and remodeling. The Journal of Cell Biology, 2011. 192(6): p. 907-917.
20. Oda, H. and M. Takeichi, Structural and functional diversity of cadherin at the adherens junction. The Journal of Cell Biology, 2011. 193(7): p. 1137-1146.
21. Suzuki, T., Regulation of intestinal epithelial permeability by tight junctions. Cellular and Molecular Life Sciences, 2013. 70(4): p. 631-659.
22. Camilleri, M., et al., Intestinal barrier function in health and gastrointestinal disease. Neurogastroenterology & Motility, 2012. 24(6): p. 503-512.
23. Van Itallie, C.M. and J.M. Anderson, Claudins and epithelial paracellular transport. Annual Review of Physiology, 2006. 68: p. 403-429.
24. Furuse, M., et al., Claudin-based tight junctions are crucial for the mammalian epidermal barrier a lesson from claudin-1–deficient mice. The Journal of Cell Biology, 2002. 156(6): p. 1099-1111.
25. Van Itallie, C., C. Rahner, and J.M. Anderson, Regulated expression of claudin-4 decreases paracellular conductance through a selective decrease in sodium permeability. The Journal of Clinical Investigation, 2001. 107(10): p. 1319-1327.
26. Amasheh, S., et al., Contribution of claudin-5 to barrier properties in tight junctions of epithelial cells. Cell and Tissue Research, 2005. 321(1): p. 89-96.

27.   Amasheh, S., et al., Na+ absorption defends from paracellular back-leakage by claudin-8 upregulation. Biochemical and Biophysical Research Communications, 2009. 378(1): p. 45-50.

28.   Amasheh, S., et al., Claudin-2 expression induces cation-selective channels in tight junctions of epithelial cells. Journal of Cell Science, 2002. 115(24): p. 4969-4976.

29.   Alexandre, M.D., Q. Lu, and Y.-H. Chen, Overexpression of claudin-7 decreases the paracellular Cl– conductance and increases the paracellular Na+ conductance in LLC-PK1 cells. Journal of Cell Science, 2005. 118(12): p. 2683-2693.

30.   Fujita, H., et al., Tight junction proteins claudin-2 and-12 are critical for vitamin D-dependent Ca2+ absorption between enterocytes. Molecular biology of the cell, 2008. 19(5): p. 1912-1921.

31.   Goodman , B.E., Insights into digestion and absorption of major nutrients in humans. Advances in Physiology Education, 2010. 34(2): p. 44-53.

32.   Reddy, I.M., N.K. Kella, and J.E. Kinsella, Structural and conformational basis of the resistance of. beta.-lactoglobulin to peptic and chymotryptic digestion. Journal of Agricultural and Food Chemistry, 1988. 36(4): p. 737-741.

33.   Hartmann, R., et al., Cytotoxic and Allergenic Potential of Bioactive Proteins and Peptides. Current Pharmaceutical Design, 2007. 13(9): p. 897-920.

34.   Fitzgerald, R.J. and H. Meisel, Milk protein-derived peptide inhibitors of angiotensin-I-converting enzyme. British Journal of Nutrition, 2000. 84(SUPPL. 1): p. S33-S37.

35.   Korhonen, H. and A. Pihlanto, Bioactive peptides: production and functionality. International Dairy Journal, 2006. 16(9): p. 945-960.

36.   Udall, J. and W. Walker, The physiologic and pathologic basis for the transport of macromolecules across the intestinal tract. Journal of Pediatric Gastroenterology and Nutrition, 1982. 1(3): p. 295-302.

37.   Reitsma, M., et al., Protein transport across the small intestine in food allergy. Molecular Nutrition & Food Research, 2014. 58(1): p. 194-205.

38.   Walker, W.A., et al., Macromolecular absorption. Mechanism of Horseradish Peroxidase Uptake and Transport in Adult and Neonatal Rat Intestine. The Journal of Cell Biology, 1972. 54(2): p. 195-205.

39.   Weaver, L., M. Laker, and R. Nelson, Intestinal permeability in the newborn. Archives of Disease in Childhood, 1984. 59(3): p. 236-241.

40.   Gardner, M.L.G., Passage of intact peptides across the intestine. Advances in the Biosciences, 1987. 65: p. 99-106.

41.   Gardner, M.L.G., Gastrointestinal absorption of intact proteins. Annual review of nutrition, 1988. 8(1): p. 329-350.

42.   Miner-Williams, W.M., B.R. Stevens, and P.J. Moughan, Are intact peptides absorbed from the healthy gut in the adult human? Nutrition Research Reviews, 2014. 27(02): p. 308-329.

43.   Yoshikawa, M., et al., Bioactive peptides derived from food proteins preventing lifestyle-related diseases. BioFactors, 2000. 12(1-4): p. 143-146.

44.   Untersmayr, E. and E. Jensen-Jarolim, The role of protein digestibility and antacids on food allergy outcomes. Journal of Allergy and Clinical Immunology, 2008. 121(6): p. 1301-1308.

45.   Bischoff, S.C., et al., Intestinal permeability – a new target for disease prevention and therapy. BMC Gastroenterology, 2014. 14(1): p. 189.

46.   Grootjans, J., et al., Non-invasive assessment of barrier integrity and function of the human gut. World Journal of Gastrointestinal Surgery, 2010. 2(3): p. 61-69.

47.   Menzies, I. Transmucosal passage of inert molecules in health and disease. in Intestinal absorption and secretion. Falk Symposium. 1984.

48.   Menzies, I., et al., Abnormal intestinal permeability to sugars in villous atrophy. Lancet, 1979. 314(8152): p. 1107-1109.

49.   Bjarnason, I., A. Macpherson, and D. Hollander, Intestinal permeability: an overview. Gastroenterology, 1995. 108(5): p. 1566-1581.

50.   Rao, A.S., et al., Urine sugars for in vivo gut permeability: validation and comparisons in irritable bowel syndrome-diarrhea and controls. American Journal of Physiology - Gastrointestinal and Liver Physiology, 2011. 301(5): p. G919-G928.

51.   Anderson, A.D., et al., Evaluation of a triple sugar test of colonic permeability in humans. Acta Physiologica, 2004. 182.

52.   van Wijck, K., et al., Novel analytical approach to a multi-sugar whole gut permeability assay. Journal of Chromatography B, 2011. 879(26): p. 2794-2801.

53. Rooyakkers, D.R., H.M.H. van Eijk, and N.E.P. Deutz, Simple and sensitive multi-sugar-probe gut permeability test by high-performance liquid chromatography with fluorescence labelling. Journal of Chromatography A, 1996. 730(1): p. 99-105.

54. Perko, M., et al., Mesenteric, coeliac and splanchnic blood flow in humans during exercise. The Journal of Physiology, 1998. 513(3): p. 907-913.

55. Van Wijck, K., et al., Exercise-induced splanchnic hypoperfusion results in gut dysfunction in healthy men. PLoS ONE, 2011. 6(7): p. e22366.

56. Øktedalen, O., et al., Changes in the gastrointestinal mucosa after long-distance running. Scandinavian Journal of Gastroenterology, 1992. 27(4): p. 270-274.

57. Ryan, A.J., R. Chang, and C.V. Gisolfi, Gastrointestinal permeability following aspirin intake and prolonged running. Medicine and Science in Sports and Exercise, 1996. 28(6): p. 698-705.

58. Van, N., Brouns, and Brummer, The effect of physical exercise on parameters of gastrointestinal function. Neurogastroenterology and Motility, 1999. 11(6): p. 431-439.

59. Van Nieuwenhoven, M.A., R.-J.M. Brummer, and F. Brouns, Gastrointestinal function during exercise: comparison of water, sports drink, and sports drink with caffeine. Journal of Applied Physiology, 2000. 89(3): p. 1079-1085.

60. Lambert, G.P., et al., Gastrointestinal permeability during exercise: effects of aspirin and energy-containing beverages. Journal of Applied Physiology, 2001. 90(6): p. 2075-2080.

61. Lambert, G., et al., Effect of aspirin and ibuprofen on GI permeability during exercise. International Journal of Sports Medicine, 2007. 28(09): p. 722-726.

62. Buckley, J.D., et al., Bovine colostrum supplementation during running training increases intestinal permeability. Nutrients, 2009. 1(2): p. 224-34.

63. Marchbank, T., et al., The nutriceutical bovine colostrum truncates the increase in gut permeability caused by heavy exercise in athletes. American Journal of Physiology - Gastrointestinal and Liver Physiology, 2011. 300(3): p. G477-G484.

64. van Wijck, K., et al., Physiology and pathophysiology of splanchnic hypoperfusion and intestinal injury during ex ercise: strategies for evaluation and prevention. American Journal of Physiology - Gastrointestinal and Liver Physiology, 2012. 303(2): p. G155-G168.

65. Van Wijck, K., et al., Aggravation of exercise-induced intestinal injury by Ibuprofen in athletes. Medicine and Science in Sports and Exercise, 2012. 44(12): p. 2257-2262.

66. Le Ferrec, E., et al., *In vitro* models of the intestinal barrier. Alternatives to Laboratory Animals, 2001. 29: p. 649-668.

67. Cogburn, J.N., M.G. Donovan, and C.S. Schasteen, A Model of Human Small Intestinal Absorptive Cells. 1. Transport Barrier. Pharmaceutical Research, 1991. 8(2): p. 210-216.

68. Chantret, I., et al., Epithelial Polarity, Villin Expression, and Enterocytic Differentiation of Cultured Human Colon Carcinoma Cells: A Survey of Twenty Cell Lines. Cancer Research, 1988. 48(7): p. 1936-1942.

69. Rousset, M., The human colon carcinoma cell lines HT-29 and Caco-2: two *in vitro* models for the study of intestinal differentiation. Biochimie, 1986. 68(9): p. 1035-1040.

70. Simon-Assmann, P., et al., *In vitro* models of intestinal epithelial cell differentiation. Cell Biology and Toxicology, 2007. 23(4): p. 241-256.

71. Walter, E., et al., HT29-MTX/Caco-2 cocultures as an *in vitro* model for the intestinal epithelium: *In vitro–in vivo* correlation with permeability data from rats and humans. Journal of pharmaceutical sciences, 1996. 85(10): p. 1070-1076.

72. Artursson, P., K. Palm, and K. Luthman, Caco-2 monolayers in experimental and theoretical predictions of drug transport. Advanced Drug Delivery Reviews, 2012.

73. Novak, E.A. and K.P. Mollen, Mitochondrial dysfunction in inflammatory bowel disease. Frontiers in cell and developmental biology, 2015. 3.

74. Crabtree, H.G., Observations on the carbohydrate metabolism of tumours. Biochemical Journal, 1929. 23(3): p. 536.

75. Marroquin, L.D., et al., Circumventing the Crabtree effect: replacing media glucose with galactose increases susceptibility of HepG2 cells to mitochondrial toxicants. Toxicological sciences, 2007. 97(2): p. 539-547.

76. Aguer, C., et al., Galactose enhances oxidative metabolism and reveals mitochondrial dysfunction in human primary muscle cells. PLoS ONE, 2011. 6(12): p. e28536-e28536.

77. Kase, E.T., et al., Remodeling of oxidative energy metabolism by galactose improves glucose handling and metabolic switching in human skeletal muscle cells. PLoS ONE, 2013. 8(4).

78. Rossignol, R., et al., Energy substrate modulates mitochondrial structure and oxidative capacity in cancer cells. Cancer research, 2004. 64(3): p. 985-993.
79. Wilson, G., et al., Transport and permeability properties of human Caco-2 cells: An *in vitro* model of the intestinal epithelial cell barrier. Journal of Controlled Release, 1990. 11(1): p. 25-40.
80. Artursson, P. and J. Karlsson, Correlation between oral drug absorption in humans and apparent drug permeability coefficients in human intestinal epithelial (Caco-2) cells. Biochemical and Biophysical Research Communications, 1991. 175(3): p. 880-885.
81. Yee, S., *In vitro* permeability across Caco-2 cells (colonic) can predict *in vivo* (small intestinal) absorption in man—fact or myth. Pharmaceutical Research, 1997. 14(6): p. 763-766.

Chapter 2

The effect of endurance exercise on intestinal integrity in well-trained healthy men

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Abstract

Exercise is one of the external factors associated with impairment of intestinal integrity, possibly leading to increased permeability and altered absorption. Here, we aimed to examine to what extent endurance exercise in the glycogen-depleted state can affect intestinal permeability toward small molecules and protein-derived peptides in relation to markers of intestinal function. Eleven well-trained male volunteers (27±4 years) ingested 40 g of casein protein and a lactulose/rhamnose (L/R) solution after an overnight fast in resting conditions (control) and after completing a dual – glycogen depletion and endurance – exercise protocol (first protocol execution). The entire procedure was repeated 1 week later (second protocol execution). Intestinal permeability was measured as L/R ratio in 5 h urine and 1 h plasma. Five hour urine excretion of betacasomorphin-7 (BCM7), postprandial plasma amino acid levels, plasma fatty acid binding protein 2 (FABP2), serum pre-haptoglobin 2 (preHP2), plasma glucagon-like peptide 2 (GLP2), serum calprotectin, and dipeptidylpeptidase-4 (DPP4) activity were studied as markers for excretion, intestinal functioning and recovery, inflammation, and BCM7 breakdown activity, respectively. BCM7 levels in urine were increased following the dual exercise protocol, in the first as well as the second protocol execution, whereas 1 h-plasma L/R ratio was increased only following the first exercise protocol execution. FABP2, preHP2, and GLP2 were not changed after exercise, whereas calprotectin increased. Plasma citrulline levels following casein ingestion (iAUC) did not increase after exercise, as opposed to resting conditions. Endurance exercise in the glycogen depleted state resulted in a clear increase of BCM7 accumulation in urine, independent of DPP4 activity and intestinal permeability. Therefore, strenuous exercise could have an effect on the amount of food-derived bioactive peptides crossing the epithelial barrier. The health consequence of increased passage needs more in depth studies.

Introduction

The intestinal epithelium is the body's largest interface between the external and internal environment, regulating fluxes of ions, water, nutrients, and other molecules while protecting the host against potentially harmful agents and invading microorganisms. The importance of a well-functioning intestinal barrier is generally supported. At the same time, far less is understood about the nature and consequences of what is generally referred to as "increased intestinal permeability" [1, 2]. However, a "leaky gut" concept as persistently used in popular media as an all-embracing cause of multiple diseases is an obvious oversimplification [1]. In fact, "leaks" in the intestinal barrier, either discretely localized or scattered along the full length of the intestines, can relate to many distinct processes, such as an impaired mucus layer, disrupted tight junctions, an attenuated immune defense, changes in intestinal cell functionality, etc. Several studies relate impairment in absorption functions of the intestinal barrier, either intentional or unintentional, to the development of certain disorders, but knowledge on the exact mechanisms is lacking. As a consequence, a demand for a more detailed understanding of intestinal functioning remains, including insight in those processes and factors involved in impairment or restoration of its barrier function.

Particular forms of physical stress, including strenuous exercise, are known to change barrier function. Intestinal integrity can be assessed by its permeability to small compounds, such as the inert sugar lactulose and by circulating markers of intestinal damage, such as fatty acid binding protein 2 (FABP2) and prehaptoglobin 2 (preHP2) [3, 4]. Several studies, although inconclusive [5, 6], have shown increased intestinal permeability to small molecules after exercise [7-10]. The lack in consistent outcomes might reflect that the various exercise studies are difficult to compare, also because they differ in type and intensity of exercise as well as in the methodology to assess intestinal permeability.

The prevalence of food-dependent exercise-induced anaphylaxis furthermore suggests that increased absorption of intact and immunologically active proteins, or their fragments, after strenuous exercise is likely [11]. Although it has been shown, to some extent, that specific protein and protein-derived peptides are detectable in blood and urine after oral ingestion in humans [11-14], limited studies have been performed on the effect of strenuous exercise on the passage of intact proteins and their fragments. This is of relevance for extrapolation to the general population, because some fragments can have specific bioactive activities or may induce adverse reactions of the immune system.

In a recent study, we used a dual exercise protocol involving both a glycogen depletion interval exercise and prolonged endurance exercise, which resulted in a clear post-exercise inflammatory response in well-trained individuals [15]. In this study, this protocol was applied to study intestinal integrity and intestinal function (digestion and enterocyte metabolic mass). The casein protein bolus ingested in this study is a source of several bioactive peptides, which are known for their resistance to digestion [16]. One of the casein-derived bioactive peptides that have been identified *in vivo* is betacasomorphin-7 (BCM7).

The aim of this study was to determine the effects of a controlled dual exercise protocol [15] on intestinal permeability to small molecules and excretion of protein-derived peptides in relation to markers of intestinal function.

Material and Methods

Subjects

Shortly after the competition season had ended, twelve well-trained healthy male cyclists were recruited via posters at the university, local sport centres, and via social media. Subjects were included with at least 2 years of cycling experience, a training frequency of at least two times a week during high season and no known records of milk allergy, immune diseases or intestinal diseases. Exclusion criteria were smoking, using hard drugs, using NSAIDs on a chronic basis, using any medication for gastric or intestinal complaints or donating blood during the last 6 weeks before the start and during the study. Subjects were requested not to perform intense physical activity 3 days prior to the test days. They were also instructed not to consume dairy-containing food products 2 days prior to and during the test days, not to use alcohol 4 days prior to and during the test days, and not to use soft drugs 2 weeks prior to and during the test days. This study was approved by the medical ethical committee of Wageningen University (METC-WU (13/10); CCMO Number NL44947.081.13), and conducted in accordance with the Declaration of Helsinki (revised version, October 2008, Seoul). Each of the subjects gave their written informed consent.

Baseline testing

Baseline testing was performed in the week prior to the start of the experimental protocol and consisted of anthropometric measurements including DEXA scan and a maximal aerobic capacity test. The maximal aerobic capacity test was adapted from [17] and performed using an electronically braked cycle ergometer (Lode Excalibur, Groningen, The Netherlands). After a short warming-up, the subjects started cycling at 100 W. Every minute the power increased with 20 W. The subjects had to cycle until they were no longer able to maintain the workload (Wmax; pedal frequency falling from 90 to 100 rpm to <70 rpm). O_2-consumption and CO_2-production were measured with an Oxycon Pro (Jaeger, Hoechberg, Germany) to define the maximal aerobic capacity, VO_2max.

Day 1 (rest)					
Overnight fast	Blood and urine sampling	Protein and sugar test solution	Blood and 5h urine sampling	Glycogen depletion test	Small high fat snack

Day 2 (post-exercise)					
Overnight fast	Small high fat snack	Endurance cycling test	Blood and urine sampling	Protein and sugar test solution	Blood and 5h urine sampling

| Day 3 (24h post-exercise)	
Overnight fast	Blood sampling

Figure 2.1. Schematic overview of experimental design.

This scheme shows the different stages of the 3-day experimental protocol (first protocol execution). This complete experimental protocol was repeated after 1 week in exactly the same way (second protocol execution).

Study design

This study had a single arm intervention design, with measurements performed during three conditions: rest, exercise, and 24 h post exercise (see **Figures 2.1 and 2.2**). Each subject acted as their own control (resting condition). The experimental protocol started with assessing intestinal permeability during the resting condition. The subjects arrived at the university after being fasted overnight (since 2300 h). A venflon cannula was inserted in the antecubital vein and blood was sampled (baseline-rest, taken as t = 0 for calculations). A baseline urine sample was also collected. This was followed by the ingestion of a multi-inert-sugar test solution (see intestinal permeability testing) and a protein bolus containing 40 g of casein protein (Kind gift of Dutch Protein & Services BV, Tiel, The Netherlands) dissolved in 400 mL of tap water. Blood and urine was sampled (with t=15 being 15 min after ingestion of casein) during the next 5 h at regular intervals, after which lunch was offered. The 5 h duration of sampling was chosen to accommodate possible slow digestion kinetics of casein [18, 19]. The exercise condition consisted of a dual exercise protocol and started in the evening following the assessment of the resting control condition. The subjects returned to the university 2 h after their regular dinner at home and completed the first part of the dual exercise intervention. This first part aimed at achieving glycogen depletion and consisted of 2-min blocks of cycling alternating between very high intensity (90% Wmax ≈ 90% VO2max), and moderate intensity (50% Wmax ≈ 55% VO2max) at a pedal frequency of 90–100 rpm [20, 21]. When they were no longer able to cycle at 90% Wmax, the intensity was reduced to 80% Wmax (still alternating with 50% Wmax), and subsequently to 70% Wmax. When alternating 70% and 50% Wmax could not be maintained, this exercise protocol was stopped. The subjects then received a small high-fat snack (Two crackers with peanut butter:

164 kCal of which 53 energy percent (En%) fat and 26 En% carbohydrates; or two crackers with pate: 260 kCal of which 61 En% fat and 32 En% carbohydrates) to reduce hunger feelings at night. They were not allowed to eat anything else until returning to the university the next morning, where they received the same high-fat snack before continuing with the second part of the dual exercise intervention. This second part consisted of 90 min of cycling at a moderate intensity of 50% Wmax, which can be considered strenuous in combination with the prior glycogen depletion part. Directly after completing the second part of the dual exercise protocol, a venflon cannula was inserted in the antecubital vein and blood was sampled (baseline-exercise, taken as t=0 for calculations). A baseline urine sample was also collected. This was again followed by the ingestion of the multi-inert-sugar test solution and the casein protein solution. Blood and urine was sampled (with t=15 being 15 min after ingestion of the casein protein solution) for 5 h at regular intervals, after which lunch was offered. The next morning, the subjects came back to the university once more (24 h post exercise condition) to donate a single fasted blood sample. This complete single arm experimental protocol was repeated after 1 week to test for reproducibility in outcomes. In the following sections, these two executions of the experimental protocol are referred to as first and second protocol execution.

Blood and urine sampling

Venous blood was sampled during rest and exercise at baseline and at 15, 30, 60, 90, 120, and 300 min after casein protein intake. In the two cases that the venflon catheter could not be successfully placed, blood was sampled by venapunction at baseline and at 60, 120, and 300 min after casein protein intake. One fasted blood sample was drawn by venapunction 24 h post exercise. Blood for the analyses was sampled in tubes (Vacutainer; Becton Dickinson, Breda, The Netherlands) containing either EDTA or EDTA supplemented with protease inhibitor cocktail (Sigma Aldrich, Zwijndrecht, The Netherlands), which were directly centrifuged at 2000 g for 10 min at 4°C, aliquoted and stored at -80°C within 20 min after sampling until further analysis. Blood was also sampled in serum separator tubes; it was left to clot in the dark for at least 30 min at room temperature, after which the tubes were centrifuged at 2000 g for 10 min at room temperature to obtain serum, which was directly aliquoted and stored at -80°C until further analysis. Collected urine was supplemented with chlorhexidine to prevent bacterial growth. During both the resting and the exercise condition, one urine sample was collected at baseline and urine was collected and pooled over a 5 h period following intake of the multi-inert-sugar and casein bolus. The total sample volume was recorded and aliquots were stored at -20°C until further analysis.
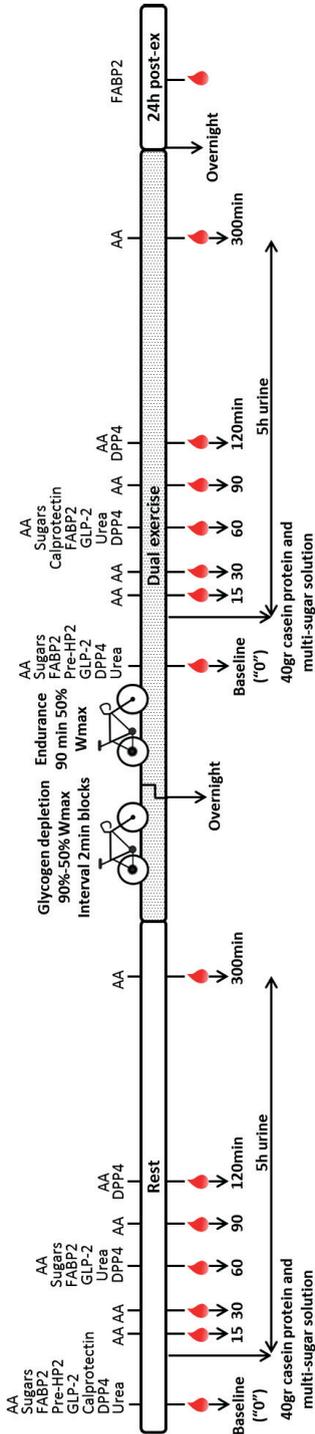
Figure 2.2. Schematic overview of blood sampling and analyses during one repetition of the experimental protocol.
This scheme shows the blood sampling during rest, exercise, and 24 h post exercise. The resting condition started at 0800 h and finished around 1400 h with lunch. The exercise condition started around 2000 h in the evening of the same day (2 h after dinner), and continued at 0730 h the next morning, and sampling lasted till around 1400 h on that day. The 24 h post exercise blood sampling took place around 0900 h the next day. This complete experimental protocol with a dual exercise protocol was repeated in exactly the same way 1 week later. Samples taken at baseline were used as t = 0 for calculations. The different blood analyses are displayed at the different sampling time points. AA: amino acids; sugars: lactulose, L-rhamnose, and D-xylose.

Parameters of intestinal permeability

To study intestinal permeability to inert, nondigestible sugar molecules, a multi-sugar test solution was used containing three sugar probes: 5 g of lactulose, 1 g of L-rhamnose, and 0.5 g of D-xylose (BCM Specials, Nottingham, UK). These sugars were dissolved in 100 mL tap water and ingested at the start of the resting condition as well as the exercise condition. To analyse urinary levels of lactulose, L-rhamnose, and D-xylose, 250 µL urine was diluted 20 times with 0.08 mmol/L $AgNO_3$ to precipitate chloride, after which the supernatant was desalted by eluting it over a Dionex Onguard Ba and H column (Thermo Fisher Scientific BV, Breda, The Netherlands). This eluate was analysed by HPAEC (Dionex-HPLC ICS5000; Thermo Fisher Scientific) using pulsed electrochemical detection. Urinary sugar probe excretion was calculated by multiplying the concentration of each of the sugars probes with urine volume, after which the baseline excretion was subtracted. Recovery of the sugar probes was expressed as percentage of the orally ingested dose. Lactulose-to-L-rhamnose (L/R) ratios were calculated from the urinary excretion levels. An increased L/R ratio implies increased intestinal permeability. Levels of lactulose, L-rhamnose, and D-xylose were also measured in EDTA plasma. 125 µL EDTA plasma was transferred to Eppendorf tubes containing a 3000 Da cut-off filter (Amicon Ultra 0.5 mL 3 K; Millipore) to remove the plasma proteins. The filter tubes were centrifuged for 30 min at 11,000 g at 4°C, and clear plasma filtrate was inserted in the cooled sample processor (233 XL; Gilson, Middleton, WI). Sugars were subsequently analysed by LC-MS as described elsewhere [22]. Plasma L/R ratios were calculated from the 1 h plasma concentrations corrected for the concentrations found at the respective baseline.

Betacasomorphin-7 and dipeptidylpeptidase-4

To measure intestinal passage of protein-derived peptides, a specific fragment of casein, betacasomorphin-7 (BCM7), was measured in baseline and 5 h urine samples, cleaned-up by centrifugation through a 30 kD cutoff filter (Amicon Ultra; Merck Millipore, Co. Cork, Ireland). Flat-bottom ELISA plates (MaxiSorp, Nunc, Roskilde, Denmark) were coated with 100 µL 1.5 µg/mL BCM7-polylysine conjugate solution. The analysis was performed in duplicate with the competitive ELISA described elsewhere [23] in which the primary anti-BCM7 polyclonal antibodies (Abnova, Taipei, Taiwan) were used in concentration of 3.4 µg/mL. Dipeptidylpeptidase-4 (DPP4; EC 3.4.14.5) is the only enzyme able to breakdown BCM7 [24]. Therefore, serum activity of DPP4 in sample taken at baseline, as well as 60 and 120 min after casein intake, were determined for its potential effect on urine BCM7 levels. The photometric method was used as described by Wasilewska et al. [25]. The calculations were made after adjusting the measurements with the blank; the enzyme activity was calculated as: 100 9 (E-C)/S (where E, C, and S stand for the absorbance of the test, control, and standard samples, respectively). One unit of the enzyme activity was defined as the amount of the enzyme liberating 1 µmol p-nitroanilide/min/L test serum at 37°C.

Intestinal damage and recovery markers

FABP2 was measured as an acute marker of small intestinal injury in EDTA plasma (1:1) with an in-house developed ELISA, as described elsewhere [26]. Serum prehaptoglobin-2 (preHP2), also known as zonulin, was measured as a marker of intestinal integrity in serum (1:20) with a commercial ELISA kit (Immundiagnostik AG, Bensheim, Germany), following the instructions of the manufacturer. This ELISA kit only detects the active, uncleaved, form of preHP2. Plasma glucagon-like peptide 2 (GLP2) [1–34] levels were measured because this compound is positively related to improvement of intestinal permeability [27]. GPL2 was measured in duplicate in EDTA plasma treated with protease inhibitor using a commercial ELISA kit (Phoenix pharmaceuticals, Karlsruhe, Germany) according to instructions of the manufacturer. Prior to testing, 200 µL of plasma was loaded onto a pretreated C18 SEP-column (Phoenix Pharmaceuticals) to remove proteins, after which the eluent was freeze-dried. The lyophilized samples were reconstituted in 125 µL assay buffer.

Inflammatory marker

Serum calprotectin is an antimicrobial protein considered to be an early parameter of systemic inflammation [28]. Calprotectin analyses were performed with the EliATM Calprotectin assay following manufacturer's instructions (14-5610-01; Thermo Fisher Scientific, Etten-Leur, The Netherlands) by a specialized medical laboratory (SHL, Etten-Leur, The Netherlands).

Amino acid and urea levels

Plasma amino acids (AAs) were analysed with HPLC after precipitating plasma proteins in 50 µL plasma with 200 µL 3% perchloric acid. Individual AAs were determined by UFLC (Shimadzu, 's-Hertogenbosch, The Netherlands) using a precolumn derivatization with ophtaldialdehyde and fluorimetric detection [29]. Urea is a primary metabolite derived from dietary protein and was measured in serum (1:100) with a commercial assay (Abcam, Cambridge, UK).

Statistics

Repeated measures ANOVA with Tukey's multiple comparison tests were used to assess changes in plasma GLP2, serum and plasma FABP2, serum preHP2 in response to exercise (exercise condition) and casein intake only (resting condition) (GraphPad Prism 6, GraphPad Software Inc., San Diego, CA). Ratio paired T-tests were used to assess changes in urinary and plasma sugars probes, L/R ratios, urinary BCM7 and serum calprotectin between resting and exercise condition (GraphPad Prism 6). When urinary BCM7 levels were too low to detect in the urine, a lower level of detection (1.0 ng/mL) was imputed to enable paired statistics. Exercise, protocol execution, and exercise x protocol execution were the fixed parameters of the mixed model with LSD estimated marginal means which was used to assess changes in AA levels and AA-level-derived incremental area under the curve (iAUC) in

response to exercise and casein intake (IBM SPSS Statistics 22, IBM Corporation, Armonk, NY).

Results

Subject characteristics

Physical parameters of the 11 male volunteers are shown in **Table 2.1**.

Table 2.1. Subject characteristics

Age (years)	26.7 ± 3.5
Height (m)	1.87 ± 6.5
Weight (kg)	77.8 ± 9.4
BMI (kg/m^2)	22.2 ± 1.5
Fat mass (% of total mass)	13.6 ± 6.1
W_{max} (W)	406.4 ± 25.4
VO_{2max} (mL/kg/min)	62.5 ± 6.0

Data are shown as mean ± SD, n = 11.

Exercise-induced intestinal damage

To assess exercise-induced effects on intestinal permeability, urinary as well as plasma recovery levels of lactulose and L-rhamnose were determined and calculated as L/R ratios. Five hour urinary lactulose, L-rhamnose, and L/R ratios were not different after exercise compared with resting conditions (**Figure 2.3**). In contrast, plasma L/R ratio was increased 1 h after exercise (P = 0.01), suggesting an increase in intestinal permeability, mainly explained by a decrease in plasma L-rhamnose (P = 0.002). However, this was only the case during the first session. During the second protocol execution, there was no change in plasma L-rhamnose and L/R ratio, suggesting no change in intestinal permeability. Urinary and plasma levels of xylose were similar for all conditions.

Urinary BCM7 levels

Urinary levels of BCM7 were increased after ingestion a single bolus of casein protein during the exercise condition compared to the resting condition (P = 0.03 for both protocol executions) (**Figure 2.4A**), suggesting increased intestinal absorption. These effects were similar for the first and second protocol execution. Serum DPP4 activity was measured, as that can have an effect on circulating BCM7 levels. There was no effect of casein protein intake on DPP4 activity and therefore average activity (baseline, T = 60 and T = 120) for the resting condition compared to the exercise condition are shown. The levels of this peptidase were similar for each of the conditions (**Figure 2.4B**) and individual BCM7 and DPP4 activity levels were not correlated (R^2 = 0.024, P = 0.38). The effect of exercise on small intestinal epithelial damage was determined by assessing levels of circulating FABP2. **Figure 2.5A** shows that plasma FABP2 levels were not increased directly after exercise compared to the resting condition, suggesting no intestinal epithelial damage had taken place. During both

protocol executions, however, FABP2 levels decreased significantly 1 h after casein intake, independent of exercise, and in both protocol executions those levels increased again to resting levels during the exercise condition and 24 h post exercise. In line with this finding, there was no change in serum preHP2 levels when comparing the resting condition with the exercise condition (**Figure 2.5C**). There was, however, a significant increase (First protocol execution P < 0.0001 and second protocol execution P = 0.008) in serum calprotectin levels 1 h after completing the dual exercise protocol (**Figure 2.5B**) compared to resting condition baseline levels, indicating the presence of inflammation. During the second protocol execution, serum GLP2, a hormone that is involved in intestinal repair, was increased after the combination of exercise and casein compared to resting condition baseline values (**Figure 2.5D**).

Plasma AA profiles

A single bolus of 40 g casein protein was ingested during the resting condition as well as during the exercise condition. Data on all AAs are in **Table 2.2**. As an effect of exercise, serum urea levels increased compared to the levels in the resting condition (**Figure 2.6**), suggesting increased AA oxidation or breakdown under these circumstances. Absolute plasma taurine levels were constantly higher during the exercise condition compared to resting conditions **(Figure 2.7H)**. The postprandial increase in total AA due to casein protein intake was diminished after exercise (iAUC; P = 0.014) (**Figure 2.7A**). This effect is mainly explained by a less pronounced increase in nonessential amino acids (nEAA) (iAUC; P < 0.001) and partly by a smaller increase in plasma essential amino acids (EAAs) (iAUC; P = 0.044) (**Figure 2.7B and C**). Within the group of nEAA, alanine had the largest contribution to the diminished increase in plasma levels (**Figure 2.7G**). A striking effect of exercise was found on the citrulline levels after casein intake (**Figure 2.7D**). During the exercise condition, the increase in citrulline was virtually absent (iAUC, P < 0.001), with a 10-fold smaller iAUC compared to resting during the first protocol execution (1216 µmol min/mL vs. 120 µmol min/mL) and an almost threefold lower iAUC compared to resting during the second protocol execution (1341 µmol min/mL vs. 488 µmol min/mL) (**Table 2.2**). Citrulline can be produced by the intestine from glutamine. The cumulative plasma levels of glutamine and glutamate (GLX), however, did not show a significantly lower postprandial iAUC after exercise (**Figure 2.7E**). Citrulline can undergo renal conversion into arginine (**Figure 2.7F**). As for citrulline, total plasma levels of arginine were lower during the exercise condition compared to the resting condition (**Table 2.2**).

A

Urinary L/R ratio

Rest / Exercise — 1st protocol execution
Rest / Exercise — 2nd protocol execution

B

Plasma L/R ratio

*

Rest / Ex 1h Cas — 1st protocol execution
Rest / Ex 1h Cas — 2nd protocol execution

C

Urinary lactose recovery (%)

Rest / Exercise — 1st protocol execution
Rest / Exercise — 2nd protocol execution

D

Plasma lactulose (µM)

Rest / Ex 1h Cas — 1st protocol execution
Rest / Ex 1h Cas — 2nd protocol execution

E

Urinary rhamnose recovery (%)

Rest / Exercise — 1st protocol execution
Rest / Exercise — 2nd protocol execution

F

Plasma rhamnose (µM)

**

Rest / Ex 1h Cas — 1st protocol execution
Rest / Ex 1h Cas — 2nd protocol execution

G

Urinary xylose recovery (%)

Rest / Exercise — 1st protocol execution
Rest / Exercise — 2nd protocol execution

H

Plasma xylose (µM)

Rest / Ex 1h Cas — 1st protocol execution
Rest / Ex 1h Cas — 2nd protocol execution

33

Figure 2.3. Effect of exercise on markers of intestinal permeability in urine and plasma

L/R ratio (A) and recoveries of lactulose (C), L-rhamnose (E), and D-xylose (G) in 5h pooled urine are shown as mean ± SEM. L/R ratio (B) and levels of lactulose (D), L-rhamnose (F), and D-xylose (H) in 1h plasma (n=10 for first protocol execution) are shown as mean ± SEM. Statistical comparisons are made between values during the resting condition (rest) and the exercise condition (Exercise and Exercise+ 1h casein protein intake) within the first protocol execution (circles) and the second protocol execution (squares). The * symbol depicts P<0.05 and the ** symbol depicts P<0.01.
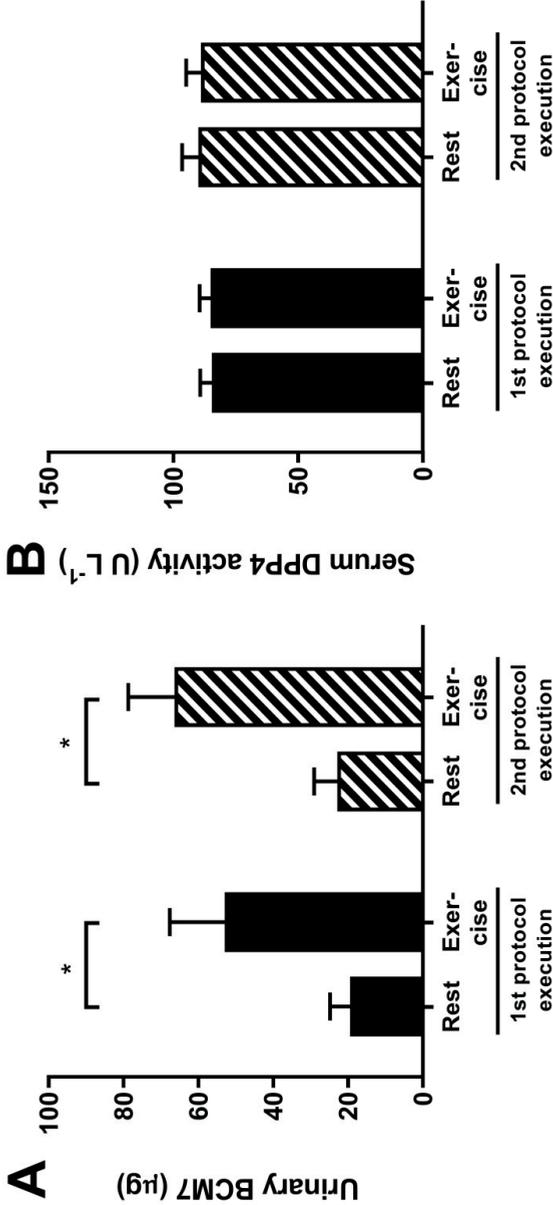

Figure 2.4. Effect of exercise and serum DPP4 activity on urine betacasomorphin-7 (BCM7) levels

Urinary BCM7 levels (A) and serum DPP4 activity (B) (n=10) are shown as mean ± SEM. Statistical comparisons are made for the first protocol execution (black) and the second protocol execution (striped) between the resting condition (Rest) and the exercise condition. The * symbol depicts P<0.05
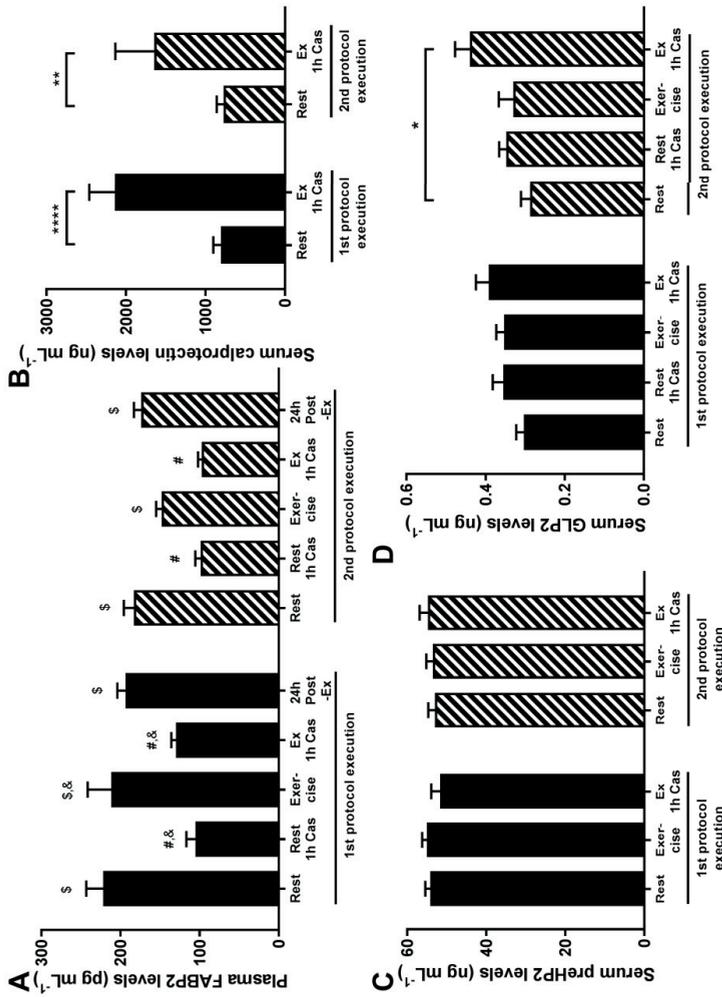
Figure 2.5. Effect of exercise on markers of intestinal integrity, recovery and systemic inflammation

Levels of plasma FABP2 (A), serum calprotectin (B), serum pre-haptoglobin 2 (preHP2) (C), and serum glucagon-like peptide 2 (GLP2) (D) are shown as mean ± SEM. Statistical comparisons are made for the first protocol execution (black) and the second protocol execution (striped) between the resting condition (rest) and the exercise condition with or without 1h casein intake (Ex 1h Cas), and for plasma FABP2 also 24h post-exercise. For serum preHP2, 1h casein sample in the resting condition was not analysed. The * symbol depicts P<0.05, ** P<0.01, and **** P<0.0001. For plasma FABP2, bars with different symbols (#, & and $) are statistically different.
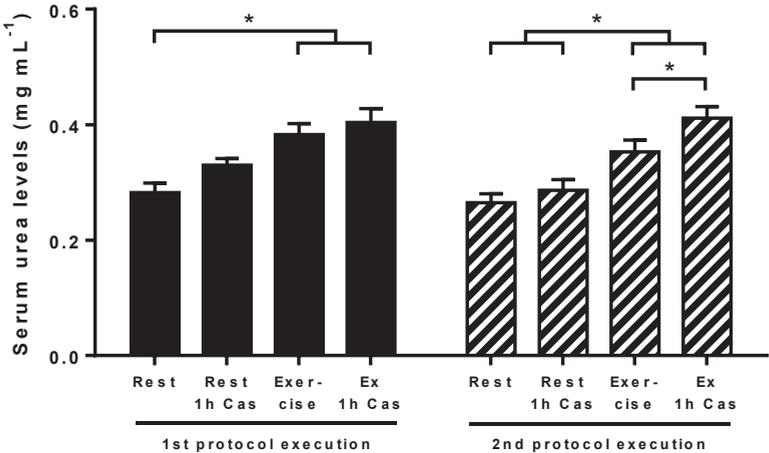
Figure 2.6. Effect of exercise and casein intake on serum urea levels
Data are shown as mean ± SEM. Serum levels during the first (black) and second (striped) protocol execution are compared between the resting condition (rest) and exercise condition in combination with 1h casein intake (Rest and Ex 1h Cas). The * symbol depicts P<0.05.

A Plasma total AA level (µmol mL⁻¹) vs Time (min)

B Plasma essential AA level (µmol mL⁻¹) vs Time (min)

C Plasma non-essential AA level (µmol mL⁻¹) vs Time (min)

D Plasma citrulline level (µmol mL⁻¹) vs Time (min)

E Plasma GLX level (µmol mL⁻¹) vs Time (min)

F Plasma arginine level (µmol mL⁻¹) vs Time (min)

G Plasma alanine level (µmol mL⁻¹) vs Time (min)

H Plasma taurine level (µmol mL⁻¹) vs Time (min)

1st protocol execution Rest
1st protocol execution Exercise
2nd protocol execution Rest
2nd protocol execution Exercise
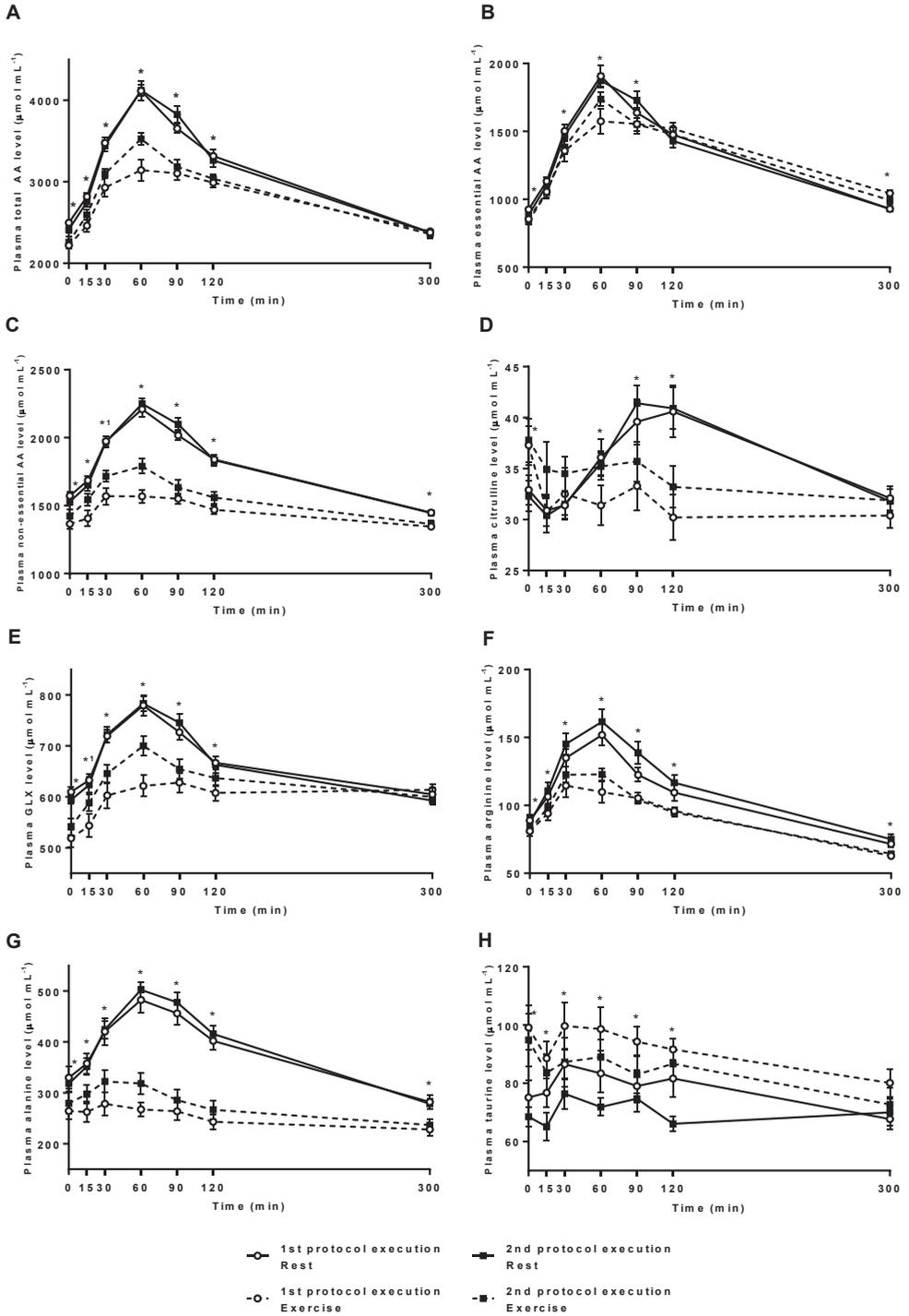
Figure 2.7. Effect of exercise on postprandial plasma amino acid levels

Plasma levels of total AAs (A), EAAs (B), nEAAs (C), citrulline (D), glutamine+ glutamate (GLX; E), arginine (F), alanine (G), and taurine (H) following casein bolus intake are shown as mean ± SEM. Comparisons are made for the first (open circles) and second (closed squares) protocol execution between the resting condition (line) versus exercise condition (dashed line). N=10 for first protocol execution. Significant differences are depicted with * when different for both protocol executions and *[1] when different for the first protocol execution only.

Table 2.2. Effect of exercise on postprandial amino acid levels (as 5h iAUC, peak height and time-to-peak)

		1st protocol execution			2nd protocol execution			1st protocol execution			2nd protocol execution			1st protocol execution			2nd protocol execution		
		Rest	Exercise	P-value	Rest	Exercise	P-value	Rest	Exercise	P-value	Rest	Exercise	P-value	Rest	Exercise	P-value	Rest	Exercise	P-value
TotAA	Mean	192.6	168.9	0.248	211.5	179.2	0.005	1668.3	1047.6	0.001	1708.6	1317.2	0.002	63.3	76.7	0.169	66.0	63.0	0.591
	SEM	20.8	14.9		11.6	15.5		135.0	104.4		87.6	116.2		3.3	8.8		4.0	5.4	
EAAs	Mean	127.9	144.2	0.206	135.7	146.1	0.009	1001.1	817.5	0.036	1009.3	933.4	0.136	63.3	76.7	0.225	72.0	66.0	0.555
	SEM	14.0	11.0		8.8	7.4		87.4	72.6		60.7	70.6		3.3	8.8		4.9	7.5	
nEAAs	Mean	66.7	27.7	0.001	77.8	39.1	<0.001	667.1	258.4	<0.001	712.1	393.1	<0.001	63.3	60.0	0.760	63.0	49.5	0.041
	SEM	7.6	5.5		4.6	6.1		54.2	39.2		36.7	53.7		3.3	11.2		3.0	7.1	
CITR	Mean	1.2	0.1	0.004	1.3	0.5	0.066	9.7	2.6	0.002	10.0	5.3	0.170	98.3	60.0	0.397	105.0	37.5	0.003
	SEM	0.3	0.1		0.3	0.2		1.8	1.3		2.1	2.5		11.5	33.5		5.0	15.0	
GLX†	Mean	18.0	25.8	0.090	20.8	24.7	0.177	179.8	138.5	0.113	190.2	164.3	0.057	63.3	116.7	0.173	66.0	60.0	0.343
	SEM	3.2	2.0		2.3	3.1		20.5	7.0		14.5	19.5		3.3	35.2		4.0	4.5	
ALA	Mean	17.4	2.0	<0.001	21.6	4.5	<0.001	158.7	28.6	<0.001	185.1	61.3	<0.001	63.3	70.0	0.834	69.0	42.0	0.010
	SEM	2.2	0.6		2.3	1.2		12.9	5.4		10.7	10.9		3.3	30.0		4.6	6.6	
ARG	Mean	5.9	3.7	0.053	8.0	3.8	<0.001	64.8	36.9	0.001	75.1	47.5	0.005	60.0	50.0	0.347	57.0	46.5	0.173
	SEM	0.9	0.5		0.7	0.2		6.9	4.1		7.3	2.7		0.0	10.0		3.0	5.7	
GLY	Mean	2.7	0.6	0.005	4.0	1.3	<0.001	42.6	14.8	0.005	62.5	29.9	0.008	56.7	25.0	0.007	60.0	36.0	0.002
	SEM	0.5	0.2		0.4	0.3		6.0	5.3		8.3	6.6		6.0	9.4		0.0	5.6	
ASX	Mean	4.4	2.3	0.011	4.5	3.0	<0.001	46.0	18.7	<0.001	44.9	28.7	0.001	63.3	73.3	0.397	66.0	55.5	0.173
	SEM	0.7	0.2		0.3	0.4		4.8	2.7		3.0	3.2		3.3	11.3		4.0	7.8	
HIS	Mean	2.4	3.1	0.242	2.8	3.2	0.181	29.5	21.2	0.040	30.9	28.2	0.101	63.3	70.0	0.559	60.0	57.0	0.591
	SEM	0.5	0.3		0.3	0.5		3.9	2.1		2.3	3.0		3.3	11.2		0.0	5.4	
ILE	Mean	15.8	24.9	<0.001	15.6	22.8	<0.001	128.5	137.8	0.365	128.9	140.5	0.053	63.3	83.3	0.050	75.0	69.0	0.343
	SEM	1.8	1.7		1.2	0.9		11.4	10.0		9.7	10.3		3.3	9.7		5.0	4.6	
LEU	Mean	28.5	41.6	0.002	28.8	38.2	<0.001	223.7	225.6	0.921	222.4	237.0	0.169	63.3	80.0	0.139	72.0	72.0	1.000
	SEM	2.9	2.7		1.8	1.7		20.5	17.3		13.5	16.7		3.3	11.2		4.9	6.6	
METH	Mean	5.5	1.0	<0.001	5.9	2.6	<0.001	44.4	13.4	<0.001	45.3	27.7	<0.001	63.3	43.3	0.022	69.0	54.0	0.052
	SEM	0.6	0.3		0.4	0.4		3.9	3.1		2.6	3.3		3.3	7.3		4.6	4.0	
LYS	Mean	20.5	15.2	0.028	23.5	16.3	<0.001	186.2	113.9	0.003	190.4	138.0	0.002	56.7	66.7	0.471	60.0	54.0	0.168
	SEM	2.9	1.9		1.8	1.3		19.7	14.5		12.9	10.2		3.3	10.9		0.0	4.0	
PHEALA	Mean	4.6	2.9	0.023	5.1	4.0	0.005	41.6	25.4	0.001	42.9	35.9	0.050	60.0	70.0	0.397	66.0	57.0	0.279
	SEM	0.5	0.4		0.3	0.4		3.4	3.1		2.0	3.4		0.0	11.2		4.0	5.4	
SER	Mean	8.0	3.4	0.012	6.9	4.3	0.043	71.6	29.9	0.003	69.0	39.9	0.011	66.7	70.0	0.782	69.0	54.0	0.096
	SEM	1.3	0.6		0.6	0.7		9.9	5.8		4.8	6.3		6.7	8.7		4.6	7.5	
TAU	Mean	2.0	0.8	0.024	1.2	0.4	0.047	20.4	11.9	0.092	16.5	7.3	0.062	53.3	93.3	0.311	75.0	43.5	0.299
	SEM	0.7	0.3		0.5	0.3		4.7	3.1		4.9	2.5		12.8	28.9		26.8	12.3	
THRE	Mean	10.5	6.0	0.008	10.9	7.6	0.001	85.3	42.0	<0.001	86.6	57.0	0.001	63.3	76.7	0.225	69.0	63.0	0.343
	SEM	1.5	0.6		0.8	0.7		8.0	5.1		5.8	5.3		3.3	10.1		4.6	5.4	

Table 2.2. Continued

		iAUC* (μmol min/mL; in thousands)						Maximum peak height (μmol/mL)						Time-to-peak (min)					
		1st protocol execution			2nd protocol execution			1st protocol execution			2nd protocol execution			1st protocol execution			2nd protocol execution		
		Rest	Exercise	P-value	Rest	Exercise	P-value	Rest	Exercise	P-value	Rest	Exercise	P-value	Rest	Exercise	P-value	Rest	Exercise	P-value
TRP	Mean	3.1	3.8	0.423	3.2	5.1	0.017	29.8	28.6	0.820	30.1	36.1	0.060	63.3	66.7	0.729	60.0	54.0	0.343
	SEM	0.4	0.7		0.3	0.5		2.0	4.6		2.3	1.9		3.3	8.3		0.0	6.0	
TYR	Mean	13.0	5.1	<0.001	14.0	7.9	<0.001	90.2	43.2	<0.001	92.6	60.9	<0.001	63.3	73.3	0.397	72.0	57.0	0.052
	SEM	1.2	0.7		1.1	0.6		7.7	7.0		6.6	4.7		3.3	10.1		4.9	5.4	
VAL	Mean	38.8	47.4	0.020	40.7	47.5	<0.001	238.5	234.5	0.818	252.8	254.1	0.845	70.0	90.0	0.022	75.0	87.0	0.223
	SEM	3.4	3.4		2.7	2.3		18.3	18.4		18.8	18.7		5.0	8.7		5.0	8.3	

N = 10 for first protocol execution; N = 11 for second protocol execution.
*iAUC in (μmol min/mL) reflects the incremental area under the curve (in thousands) over a period of 5 h following casein protein bolus intake.
†GLX is the cumulative of glutamine and glutamic acid levels.

Discussion

Passage of betacasomorphin-7 (BCM7)

Despite an apparently small increase in sugar permeability after exercise as seen from the classical L/R sugar test, exercise clearly increased the excretion of the casein-derived peptide, BCM7, in 5 h urine. The urinary concentrations of this fragment were elevated after exercise during both protocol executions. These findings indicate that the intestinal passage of proteins and (or) their fragments- peptides is more prone to effects of exercise compared to the absorption of sugars, which may be due to different mechanisms being involved. The appearance of these peptides is probably not facilitated via the paracellular pathway, as the absence of changes in lactulose indicates unchanged paracellular transport. Decreased L-rhamnose and unchanged D-xylose, respectively, indicate a decreased or unaltered, but not increased, noncarrier mediated transcellular transport. Finally, the increase in BCM7 cannot be directly explained by a difference in breakdown by dipeptidylpeptidase- 4 (DPP4), because there was a similar systemicDPP4 activity in both conditions. BCM7 is a bioactive peptide which can act as an opioid receptor agonist. BCM7 has also been implicated in several beneficial and adverse health effects, reviewed in detail by the European Food Safety Authority [30] and Haq et al. [31]. Almost all studies on the effects of BCM7, however, have been performed *in vitro* or with animal models. Evidence of health effects of BCM7 in humans is limited to epidemiological data. The EFSA therefore concluded that a causal relationship between BCM7 exposure and chronic disease risk could not be supported by the existing data. Our data provide an answer to one of the knowledge gaps presented by the EFSA [30] by showing that casein-derived peptides can indeed be transferred intact across the intestinal barrier. We have also attempted to measure levels of casein and casein-derived peptides in the plasma with a casein and beta-casein sandwich ELISA as well as HPLC MS/MS techniques, but we were unable to obtain the required detection sensitivity and specificity. Endurance exercise in the glycogen depleted state resulted in increased levels of BCM7 in urine after casein consumption, but whether and how these levels could subsequently amplify either beneficial or adverse health effects, requires further investigation.

Effects on intestinal integrity

Our dual exercise protocol did not induce similar effects on aspects of intestinal integrity as was found in previous studies. For example, FABP2, an acute enterocyte damage marker, was not affected in this study, while van Wijck et al. [7] found a clear increase in plasma FABP2 levels during and directly after exercise. The cause for the initial decrease in FABP2 levels following 1 h of casein intake in this study has recently been investigated. We figured out that cycling, the usual way of transportation, in a fasted condition to the test location already results in increased levels of FABP2 (unpublished data- chapter 5). This may explain

our results, as a baseline blood sample was drawn directly after arrival at the test location. The FABP2 level determined after 1 h rest likely reflects the "actual" individuals' baseline level. No additional effect of the endurance exercise was found. This may be due to the lower cycling intensity of 50% Wmax used in our protocol compared to 70% Wmax in van Wijck et al. [7], as data were obtained with the same assay. It should be noted, however, that the exercise protocol used in this study can still be regarded as intensive, which is for example reflected by the release of calprotectin from skeletal muscle [28]. Another explanation could be that the intensive glycogen depletion exercise phase the evening before the endurance exercise phase may have had a preconditioning, and even protective effect, thereby influencing the effects of the endurance exercise phase the next day. Preconditioning effects are seen following antioxidant supplementation [32] or with preoperative clamping [33]. The latter activates repair mechanisms in the gut and results in reduced damage from ischemia during the surgery itself. In this study, it was chosen to handle the dual exercise protocol as a single intervention. It would have been also interesting to study the responses evoked by each of the two phases of exercise separately.

Changes in AA profiles after casein intake

The increase in total plasma AA levels after casein intake was much lower after exercise when compared to the resting condition. As only venous blood was sampled, AA levels may have been influenced by organ uptake and release. Lower levels of total AA levels in plasma could be due to a combination of decreased digestion and absorption in the intestine and more rapid clearance resulting from tissue uptake (e.g., liver and muscle) as source for gluconeogenesis and/or for protein synthesis [34]. Casein is able to promote postprandial protein deposition [19], which is beneficial in an exercise condition. Decreased gastric emptying due to the endurance exercise would also lower plasma AA levels. It has, however, been shown that gastric emptying during exercise at the applied intensity occurs at a rate similar [35, 36] or faster than during rest [37], depending on the meal. The lower plasma total AA levels after exercise were mainly explained by lower levels of nonessential amino acids (nEAA), in particular of alanine. This effect on nEAA levels was not observed in a previous study with resistance-type exercise, in which mainly the postprandial essential amino acid (EAA) levels were lowered, whereas postprandial nEAA levels were not affected [38]. A possible explanation for this finding could again lie in the protocol set-up aimed at glycogen depletion. Gluconeogenesis is an important process during fasting, especially in combination with glycogen depletion, in order to maintain blood glucose levels [39, 40]. The most important AA precursor for gluconeogenesis is alanine, which is also the AA of which systemic levels decrease most rapidly during fasting [41]. An exception to the decrease in circulating AAs was the higher plasma levels of taurine after exercise compared to those following resting. Taurine can be released upon muscle contraction [39, 42], which likely explains this finding.

Intestinal metabolic capacity

Circulating citrulline is often used as a clinical measure of total intestinal metabolic mass [43]. The absence of an increase in circulating citrulline after ingestion of casein following the dual exercise protocol could reflect a reduced intestinal metabolic capacity. Two important AAs in citrulline metabolism are glutamine and arginine, as a precursor and product of citrulline, respectively. Depletion of glutamine, which is the main fuel for enterocytes, results in plasma citrulline depletion [44, 45]. Findings in mice suggest that during shortage of available glutamine, enterocytes possibly prioritize its utilization for energy over the production of citrulline [46]. Therefore, during periods of energy stress (i.e., fasting and exercise) the glutamine taken up by the intestine may not be converted into citrulline. Our results suggest that after strenuous exercise the intestine's energy stores or metabolic capacity may be insufficient to convert available glutamine from a casein protein bolus into citrulline. This agrees with the parallel decrease in arginine. Metabolic flux studies, with stable isotopes could give more insight in the intestinal uptake and conversion of available glutamine.

Conclusion

In conclusion, we show that the presented dual exercise protocol, combining glycogen depletion and endurance exercise, induces a clear increase in the casein-derived BCM7 peptide ending up in the urine, independent of paracellular and noncarrier-mediated intestinal permeability or DPP4 activity. This shows that strenuous exercise could have an effect on the amount of food-derived bioactive peptides crossing the epithelial barrier. The health consequences of increased passage of these peptides need more in depth studies to determine whether and what effects can occur at the levels found.

References

1. Quigley, E.M.M., Leaky gut - concept or clinical entity? Current opinion in gastroenterology, 2016. 32(2): p. 74-79.
2. Bischoff, S.C., et al., Intestinal permeability – a new target for disease prevention and therapy. BMC Gastroenterology, 2014. 14(1): p. 189.
3. Pelsers, M.M.A.L., W.T. Hermens, and J.F.C. Glatz, Fatty acid-binding proteins as plasma markers of tissue injury. Clinica Chimica Acta, 2005. 352(1–2): p. 15-35.
4. Wang, W., et al., Human zonulin, a potential modulator of intestinal tight junctions. Journal of cell science, 2000. 113(24): p. 4435-4440.
5. Nieuwenhoven, V., The effect of physical exercise on parameters of gastrointestinal function. Neurogastroenterology & Motility, 1999. 11(6): p. 431-439.
6. Ryan, A.J., R. Chang, and C.V. Gisolfi, Gastrointestinal permeability following aspirin intake and prolonged running. Medicine and science in sports and exercise, 1996. 28(6): p. 698-705.
7. Van Wijck, K., et al., Exercise-induced splanchnic hypoperfusion results in gut dysfunction in healthy men. PloS one, 2011. 6(7): p. e22366.
8. Marchbank, T., et al., The nutriceutical bovine colostrum truncates the increase in gut permeability caused by heavy exercise in athletes. American Journal of Physiology: Gastrointestinal and Liver Physiology, 2011. 300(3): p. G477-G484.
9. Pals, K.L., et al., Effect of running intensity on intestinal permeability. Journal of Applied Physiology, 1997. 82(2): p. 571-576.
10. Øktedalen, O., et al., Changes in the gastrointestinal mucosa after long-distance running. Scandinavian Journal of Gastroenterology, 1992. 27(4): p. 270-274.
11. Matsuo, H., et al., Exercise and aspirin increase levels of circulating gliadin peptides in patients with wheat-dependent exercise-induced anaphylaxis. Clinical & Experimental Allergy, 2005. 35(4): p. 461-466.
12. Sokolov, O., et al., Autistic children display elevated urine levels of bovine casomorphin-7 immunoreactivity. Peptides, 2014. 56: p. 68-71.
13. Chabance, B., et al., Casein peptide release and passage to the blood in humans during digestion of milk or yogurt. Biochimie, 1998. 80(2): p. 155-165.
14. Castell, J.V., et al., Intestinal absorption of undegraded proteins in men: presence of bromelain in plasma after oral intake. American Journal of Physiology - Gastrointestinal and Liver Physiology, 1997. 273(1): p. G139-G146.
15. Carol, A., et al., Bovine Colostrum Supplementation's Lack of Effect on Immune Variables During Short-Term Intense Exercise in Well-Trained Athletes. International journal of sport nutrition and exercise metabolism, 2011. 21(2): p. 135.
16. Segura-Campos, M., et al., Bioavailability of bioactive peptides. Food Reviews International, 2011. 27(3): p. 213-226.
17. Storer, T.W., J.A. Davis, and V.J. Caiozzo, Accurate prediction of VO2max in cycle ergometry. Med Sci Sports Exerc, 1990. 22(5): p. 704-12.
18. Tang, J.E., et al., Ingestion of whey hydrolysate, casein, or soy protein isolate: effects on mixed muscle protein synthesis at rest and following resistance exercise in young men. Journal of Applied Physiology, 2009. 107(3): p. 987-992.
19. Boirie, Y., et al., Slow and fast dietary proteins differently modulate postprandial protein accretion. Proceedings of the National Academy of Sciences, 1997. 94(26): p. 14930-14935.
20. Pollock, M.L., et al., ACSM position stand: the recommended quantity and quality of exercise for developing and maintaining cardiorespiratory and muscular fitness, and flexibility in healthy adults. Med Sci Sports Exerc, 1998. 30(6): p. 975-991.
21. Arts, F. and H. Kuipers, The relation between power output, oxygen uptake and heart rate in male athletes. International journal of sports medicine, 1994. 15(05): p. 228-231.
22. Van Wijck, K., et al., Novel analytical approach to a multi-sugar whole gut permeability assay. Journal of Chromatography B, 2011. 879(26): p. 2794-2801.

23. Sienkiewicz-Szłapka, E., et al., Contents of agonistic and antagonistic opioid peptides in different cheese varieties. International Dairy Journal, 2009. 19(4): p. 258-263.

24. Kreil, G., et al., Studies of the enzymatic degradation of β-casomorphins. Life Sciences, 1983. 33(s1): p. 137-140.

25. Wasilewska, J., et al., The exogenous opioid peptides and DPPIV serum activity in infants with apnoea expressed as apparent life threatening events (ALTE). Neuropeptides, 2011. 45(3): p. 189-195.

26. Van Wijck, K., et al., Aggravation of exercise-induced intestinal injury by Ibuprofen in athletes. Medicine and science in sports and exercise, 2012. 44(12): p. 2257-2262.

27. Benjamin, M., et al., Glucagon-like peptide-2 enhances intestinal epithelial barrier function of both transcellular and paracellular pathways in the mouse. Gut, 2000. 47(1): p. 112-119.

28. Mortensen, O.H., et al., Calprotectin is released from human skeletal muscle tissue during exercise. The Journal of physiology, 2008. 586(14): p. 3551-3562.

29. Terrlink, T., P. Van Leeuwen, and A. Houdijk, Plasma amino acids determined by liquid chromatography within 17 minutes. Clinical chemistry, 1994. 40(2): p. 245-249.

30. EFSA, Scientific Report of EFSA prepared by a DATEX Working Group on the potential health impact of β-casomorphins and related peptides. 2009, EFSA Scientific Report. p. 1-107.

31. Haq, M.R.u., et al., Impact of Milk Derived β-Casomorphins on Physiological Functions and Trends in Research: A Review. International Journal of Food Properties, 2014. 17(8): p. 1726-1741.

32. Ristow, M., et al., Antioxidants prevent health-promoting effects of physical exercise in humans. Proceedings of the National Academy of Sciences of the United States of America, 2009. 106(21): p. 8665-8670.

33. Hausenloy, D.J. and D.M. Yellon, Preconditioning and postconditioning: underlying mechanisms and clinical application. Atherosclerosis, 2009. 204(2): p. 334-341.

34. Ivy, J.L., Regulation of muscle glycogen repletion, muscle protein synthesis and repair following exercise. Journal of sports science & medicine, 2004. 3(3): p. 131.

35. Carrio, I., et al., Gastric emptying in marathon runners. Gut, 1989. 30(2): p. 152-155.

36. Murray, R., The Effects of Consuming Carbohydrate-Electrolyte Beverages on Gastric Emptying and Fluid Absorption During and Following Exercise. Sports Medicine, 1987. 4(5): p. 322-351.

37. Moore, J.G., F.L. Datz, and P.E. Christian, Exercise increases solid meal gastric emptying rates in men. Digestive Diseases and Sciences, 1990. 35(4): p. 428-432.

38. van Wijck, K., et al., Dietary protein digestion and absorption are impaired during acute postexercise recovery in young men. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 2013. 304(5): p. R356-R361.

39. Ishikura, K., S.-G. Ra, and H. Ohmori, Exercise-induced changes in amino acid levels in skeletal muscle and plasma. The Journal of Physical Fitness and Sports Medicine, 2013. 2(3): p. 301-310.

40. Henriksson, J., Effect of exercise on amino acid concentrations in skeletal muscle and plasma. Journal of experimental biology, 1991. 160(1): p. 149-165.

41. Felig, P., et al., Amino acid metabolism during prolonged starvation. Journal of Clinical Investigation, 1969. 48(3): p. 584-594.

42. Cuisinier, C., et al., Role of taurine in osmoregulation during endurance exercise. European journal of applied physiology, 2002. 87(6): p. 489-495.

43. Crenn, P., B. Messing, and L. Cynober, Citrulline as a biomarker of intestinal failure due to enterocyte mass reduction. Clinical nutrition, 2008. 27(3): p. 328-339.

44. Blachier, F., et al., Metabolism and functions of l-glutamate in the epithelial cells of the small and large intestines. The American Journal of Clinical Nutrition, 2009. 90(3): p. 814S-821S.

45. Rougé, C., et al., Manipulation of citrulline availability in humans. American Journal of Physiology-Gastrointestinal and Liver Physiology, 2007. 293(5): p. G1061-G1067.

46. Sokolović, M., et al., Fasting induces a biphasic adaptive metabolic response in murine small intestine. BMC genomics, 2007. 8(1): p. 361.

Chapter 3

Adaptation of exercise-induced stress in well-trained healthy young men

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New Findings

- What is the central question of this study?

Exercise is known to induce stress-related physiological responses, such as changes in intestinal barrier function. Our aim was to determine the test–retest repeatability of these responses in well-trained individuals.

- What is the main finding and its importance?

Responses to strenuous exercise, as indicated by stress-related markers such as intestinal integrity markers and myokines, showed high test–retest variation. Even in well-trained young men an adapted response is seen after a single repetition after 1 week. This finding has implications for the design of studies aimed at evaluating physiological responses to exercise.

Abstract

Strenuous exercise induces different stress-related physiological changes, potentially including changes in intestinal barrier function. In the Protégé Study (ISRCTN14236739; www.isrctn.com), we determined the test–retest repeatability in responses to exercise in well-trained individuals. Eleven well-trained men (27 ± 4 years old) completed an exercise protocol that consisted of intensive cycling intervals, followed by an overnight fast and an additional 90 min cycling phase at 50% of maximal workload the next morning. The day before (rest), and immediately after the exercise protocol (exercise) a lactulose and rhamnose solution was ingested. Markers of energy metabolism, lactulose-to-rhamnose ratio, several cytokines and potential stress-related markers were measured at rest and during exercise. In addition, untargeted urine metabolite profiles were obtained. The complete procedure (Test) was repeated 1 week later (Retest) to assess repeatability. Metabolic effect parameters with regard to energy metabolism and urine metabolomics were similar for both the Test and Retest period, underlining comparable exercise load. Following exercise, intestinal permeability (1 h plasma lactulose-to-rhamnose ratio) and the serum interleukin-6, interleukin-10, fibroblast growth factor-21 and muscle creatine kinase concentrations were significantly increased compared with rest only during the first test and not when the test was repeated. Responses to strenuous exercise in well-trained young men, as indicated by intestinal markers and myokines, show adaptation in Test–Retest outcome. This might be attributable to a carry-over effect of the defence mechanisms triggered during the Test. This finding has implications for the design of studies aimed at evaluating physiological responses to exercise.

Introduction

The importance of a properly functioning intestinal barrier, serving as a gatekeeper that allows the absorption of nutrients and provides a line of defence against a variety of micro-organisms and harmful molecules, is generally supported. Nevertheless, our understanding of the nature, regulatory mechanisms involved and consequences of what is generally referred to as 'increased intestinal permeability' is far from complete [1, 2]. As a consequence, there is a demand for methodology to measure and understand intestinal functioning in more detail. Exercise-induced changes in gut barrier function have been studied in several experimental settings [3-8]. Some of these studies have found an increase in intestinal permeability following exercise, whereas others have not. The same holds true for other exercise-induced parameters, such as the novel fibroblast growth factor-21 (FGF21). This factor has been shown to increase [9] or remain unchanged [10, 11] following acute exercise. The same ambiguity is found for the effect of exercise training on resting levels of FGF21 [10, 12]. Inconsistencies in finding significant outcomes for these parameters are at least partly attributable to large variations in study set-up, such as participant characteristics, type and number of exercise sessions, and exercise intensity. This makes it difficult to draw final conclusions on the effects of exercise. The differences in outcome suggest a requirement for more in-depth research on the relationship between subjects, stressors and repeated bouts of exercise. Exercise performance is known to be trainable [13, 14], but it is not entirely clear whether metabolic stress or tissue damage responses, such as increased intestinal permeability, are reproducible with repeated bouts of exercise in well-trained individuals who are used to strenuous exercise.

Insight into the test–retest variation of stress responses that are elicited by exercise may not only help to explain inconsistencies observed between different study results, but is also relevant to optimize exercise strategies for athletes and individuals with gastrointestinal disorders. In the present study, we examined the test–retest repeatability of well-trained individuals by exposing them to a strenuous exercise protocol for two consecutive weeks and studying markers of metabolic and cellular stresses and some immunological parameters. Hence, we applied a strenuous exercise protocol that comprised cycling in glycogen-depleted conditions, which has previously been shown to result in a significant immune response in trained individuals [15].

Methods

Ethical approval

This study was approved by the medical ethical committee of Wageningen University (METC-WU; ISRCTN14236739) and conducted in accordance with the Declaration of Helsinki (revised version, October 2008, Seoul). All subjects gave their written informed consent.

Subjects

Twelve well-trained healthy male cyclists were recruited via posters at the university and local sport centres and via social media shortly after the end of the cycling competition season. Subjects were included if they had at least 2 years of competitive cycling experience, trained at least two times a week during the competition season and had no known records of milk allergy, immune diseases or intestinal diseases. Exclusion criteria were smoking, use of drugs such as heroin, cocaine, and ecstasy, use of non-steroidal anti-inflammatory drugs on a chronic basis, or use of any medication for gastric or intestinal complaints. None of the subjects donated blood during the last 6 weeks before the start of the study. Subjects had to fill out a training diary, which validated that they only performed low-intensity (50% maximal aerobic capacity) endurance training sessions during the study period, but no intense physical activity, such as competing in a race or performing a vigorous training session, for 3 days prior to the test days and between the two testing periods. They were also instructed not to use alcohol for 4 days prior to and during the test days and not to use cannabis products, such as hashish and marijuana, for 2 weeks prior to and during the test days. None of the subjects used non-steroidal anti-inflammatory drugs for 2 days prior to and during both testing periods. Data on baseline characteristics and the training volume of each of the subjects are shown in **Table 3.1**.

Table 3.1. Subject characteristics

Identity	Age (years)	Body mass index (kg m^{-2})	Fat mass (% of total mass)	Maximal workload (W)	Maximal aerobic capacity (ml kg^{-1} min^{-1})	Training volume (h week^{-1})	
						Cycling	Other
1	27	21.1	6.5	400	62.2	4	1
2	31	24.9	26.7	370	47.8	6	3
3	29	23.7	21.0	400	64.3	3	2
4	33	21.0	14.0	380	66.8	6	3
5	24	21.9	12.7	430	62.4	9	—
6	22	22.6	15.2	430	65.5	12	—
7	22	21.0	13.4	400	70.2	8	1.5
8	25	20.7	5.1	380	67.0	6	6
9	29	22.3	11.4	430	58.1	9	3
10	26	21.4	10.6	400	64.0	9	1.5
11	26	24.5	13.1	450	59.6	6	6
Mean	27	22.2	13.6	406	62.5	7.1	3.0
SD	4	1.5	6.1	25	6.0	2.6	1.9

Other endurance training consisted of running and swimming, among other training. Twelve subjects were recruited, but only 11 of those subjects were used for further analysis. One subject was excluded for non-compliance with the dietary guidelines.

Pre-study assessment

Pre-study testing was performed 1 week prior to the start of the experimental period and consisted of anthropometric measurements, including a dual-energy X-ray absorptiometry scan and a maximal aerobic capacity test. The maximal aerobic capacity test was performed using an electronically braked cycle ergometer (Lode Excalibur, Groningen, The Netherlands). After a short warm-up, the subjects started cycling at 100 W, and every minute a 20 W increase was applied. The subjects had to cycle until they were no longer able to maintain the workload (Wmax). Oxygen consumption and CO_2 production were

measured with an Oxycon Pro (Jaeger, Hoechberg, Germany) to define the maximal aerobic capacity.

Study design

This intervention study consisted of a repeated single arm, before-and-after intervention design, with each subject acting as their own control. The complete experimental protocol (Test) consisted of three phases: (i) rest; (ii) exercise; and (iii) 24 h postexercise (**Figure 3.1**). The subjects arrived fasted on the first day (Rest conditions), and a blood sample was taken (time t = 0). They ingested a non-digestible multisugar test solution (*see 'Sugar intestinal permeability test'*), shortly followed by a protein shake containing 40 g of casein protein (kind gift of Dutch Protein & Services BV, Tiel, The Netherlands) dissolved in 400 ml of tap water. Blood was sampled after 1 h, and subjects received lunch. The same testing was done after a dual exercise protocol described by Carol et al. [15]. In short, the subjects came back to the university in the evening of the first day, 2 h after having their dinner at home. They were asked to stick to their usual eating habits. They completed a glycogen depletion exercise consisting of 2 min blocks of cycling, alternating between 90 and 50% Wmax at a pedal frequency of 90–100 r.p.m. When the subjects were no longer able to cycle at 90% Wmax (<70 r.p.m), the intensity was reduced to 80% Wmax (still alternating with 50% Wmax) and, subsequently, 70% Wmax. When alternating 70 and 50% Wmax could not be maintained (<70 r.p.m), the exercise was stopped. The subjects then received one of two small high-fat snacks (snack 1, 164 kCal, with 53% energy as fat and 26% energy as carbohydrate, chosen by four participants; or snack 2, 260 kCal, with 61% energy as fat and 32% energy as carbohydrate, chosen by eight participants) to reduce feelings of hunger at night. The next day, the subjects came in fasted and received the same high-fat snack as before, after which they had to cycle for 90 min at 50% Wmax. Directly after completing the second part of the exercise protocol, a blood sample (time t = 0) was taken. Next, the non-digestible multisugar test solution was ingested, shortly followed by the protein shake. Blood was sampled after 1 h, and the subjects received lunch. Finally, subjects came back the next morning (24 h postexercise) to donate a single fasted blood sample before receiving breakfast. This 3 day experimental protocol was repeated 1 week later (Retest) to evaluate the test–retest variation in the metabolic and stress/damage responses provoked by the dual exercise protocol. From now on, these two intervention periods are referred to as Test and Retest. Subjects were not allowed to switch between the two types of high-fat snack between the Test and Retest.
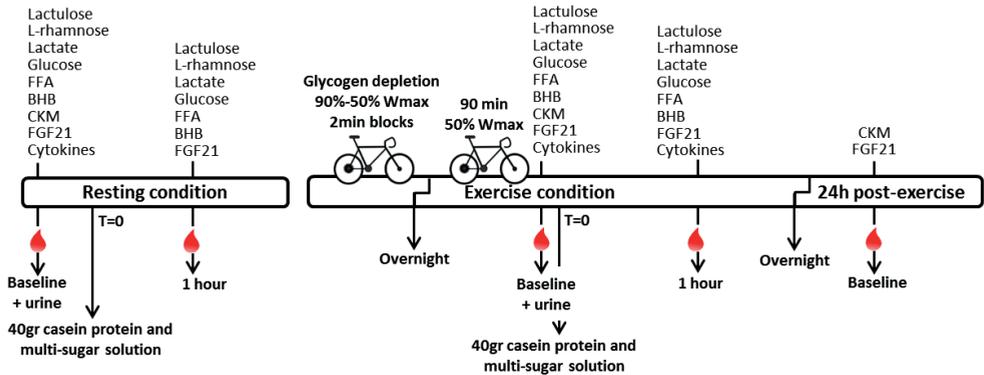
Figure 3.1. Schematic overview of blood sampling and analyses

This scheme shows the dual exercise protocol and blood sampling during rest, exercise and 24 h postexercise (Test). This complete experimental protocol was repeated in exactly the same way 1 week later (Retest). The blood analyses performed at the different sampling time points are listed. Abbreviations: BHB, β-hydroxybutyrate; CKM, creatine kinase, muscle-specific; FFA, free fatty acids; FGF21, fibroblast growth factor 21; and Wmax, maximal workload.

Blood and urine sampling

During both rest and exercise, blood was sampled from the antecubital vein before (at t = 0) and 1 h after the intake of the sugar test solution and the casein protein shake. At 24 h postexercise, only a fasted blood sample was drawn. Blood was sampled in tubes (Vacutainer; Becton Dickinson, Breda, The Netherlands) containing either EDTA or NaF, which were directly centrifuged at 2000g for 10 min at 4°C, aliquoted and stored at −80°C within 20 min after sampling until further analysis. Blood was also sampled in serum separator tubes. It was left to clot in the dark for at least 30 min at room temperature, after which the tubes were centrifuged at 2000g for 10 min at room temperature to obtain serum, which was directly aliquoted and stored at −80°C until further analysis. During both rest and exercise, urine samples were collected before the intake of the sugar test solution and the casein protein shake, and supplemented with chlorhexidine to prevent bacterial growth. Total sample volumes were recorded, and aliquots were stored at −20°C until further analysis.

Untargeted urine metabolomics

Untargeted urine metabolomics was performed on samples collected before the intake of the multisugar test solution and protein shake at rest and following exercise. Urine samples were diluted with acetonitrile (1:1 ratio) and centrifuged. Hydrophilic interaction liquid chromatography-high resolution mass spectrometry (HILIC-HRMS) experiments were performed on a QExactive system (Thermo Electron Corporation, Waltham, MA, USA) consisting of a Dionex 3000 UltiMate RS autosampler, Dionex 3000 UltiMate UHPLC pump and a QExactive mass detector. The system is controlled using Thermo Fisher Scientific

XcaliburTM version 2.2 SP1.48 software. High-resolution full MS detection was carried out using heated electrospray ionization (HESI) in both the positive and negative ionization modes using a scan range from m/z 100–1000 with the resolution set at 17,500. Prior to analysis of the study samples, the HRMS system was successfully calibrated in the positive and negative ionization mode to obtain high mass accuracy. Liquid chromatography–mass spectrometry (LC-MS) profiling was performed using hydrophilic interaction liquid chromatography and a 13.5 min gradient from 10 mM ammonium formate in 95% acetonitrile to 10 mM ammonium formate in 50% acetonitrile. Peak detection and matching across LC-MS profiles were performed using the Rpackage XCMS version 1.30.3, based on the centWave method [16]. The resulting peak intensity table was processed using MetaboAnalyst 3.0 [17]. The number of features was reduced based on the interquartile range, and normalization to constant sum and auto scaling were applied. The resulting data table was used for principal component analysis.

Muscle-related parameters

Muscle-specific creatine kinase (CKM) released in response to exercise was measured using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Ab185988; Abcam). Serum samples were diluted 200–8000 times with sample diluent in order to be able to interpolate the values from the standard curve. Concentrations of FGF21 in serum and plasma in response to exercise were measured with a commercial sandwich ELISA assay kit (DF2100; R&D Systems, Wiesbaden, Germany) according to instructions provided by the manufacturer.

Sugar intestinal permeability test

The sugar solution for studying small intestinal permeability contained the following three inert, non-digestible sugar probes: 5 g of lactulose, 1 g of L-rhamnose and 0.5 g of D-xylose (BCM Specials, Nottingham, UK). These were dissolved in 100 ml of tap water. Concentrations of lactulose and L-rhamnose were measured to determine intestinal permeability [18, 19] in EDTA plasma sampled at t=0 and 1 h after the intake of the sugar test solution and the protein shake during rest and exercise. To remove plasma proteins, EDTA plasma (125 µl) was transferred to Eppendorf tubes containing a 3000 Da cut-off filter (Amicon Ultra 0.5 ml 3 K; Millipore, Cork, Ireland). The tubes were centrifuged for 30 min at 11,000g and 4°C, and the clear plasma filtrate was inserted in the cooled sample processor (233 XL; Gilson, Middleton, WI, USA). Sugars were subsequently analysed by LC-MS as described elsewhere [20]. Plasma lactulose-to-rhamnose (L/R) ratios were calculated from the 1 h plasma concentrations and corrected for the concentrations found at t = 0.

Immune markers

Plasma concentrations of fractalkine (also known as CX3CL1), in response to exercise, were measured with a commercial sandwich ELISA assay kit (DCX310; R&D Systems) according to instructions provided by the manufacturer. Immune inflammation-related proteins [interleukin (IL)-1α, IL-1β, IL-1 receptor antagonist (RA), IL-2, IL-5, IL-6, IL-10, IL-12, IL-13, IL-25, tumour necrosis factor-α (TNF-α), interferon-γ (IFN-γ), IFN-γ- induced protein 10 (IP-10), tumor necrosis factor superfamily member 14 (LIGHT), thymic stromal lymphopoietin (TSLP) and granulocyte-colony stimulating factor (G-CSF)] were determined in serum samples by an in-house developed multiplex technology (xMAP; Luminex), according to the Luminex instructions, as previously described [21]. Non-specific heterophilic binding of immunoglobulins was prevented with HeteroBlock (Omega Biologicals, Bozeman, MT, USA), and no cross-reactivity was observed. Acquisition was performed with a Bio-Rad FlexMAP3D in combination with xPONENT software version 4.1 (Luminex). Data analysis was performed with Bioplex Manager 6.1.1 (Bio-Rad, Veenendaal, The Netherlands). For each of the cytokines, a standard curve was taken along on each of the sample plates. When sample levels measured were lower than the lower limit of quantification (LLOQ), the values were set at 10% of this cut-off value to enable statistical analyses.

Statistics

Linear mixed model analysis with Bonferroni multiple comparison tests were used to assess changes in the concentrations of all compounds analysed, except plasma L/R ratios (IBM SPSS Statistics 22, IBM Corporation, Armonk, NY). Per protocol analyses were performed. Student's paired t tests were used to assess changes in plasma L/R ratios between the rest and exercise conditions (IBM SPSS Statistics 22). Two-way ANOVA with Bonferroni post hoc testing was used to assess changes in RER and energy expenditure over time and between the rest and exercise conditions (GraphPad Prism 6, GraphPad Software Inc., San Diego, CA).

Student's paired t test with a statistical significance of one-tailed P < 0.05 was used to test whether the effect size of CKM, FGF21 and L/R ratio was decreased during the Retest compared with the Test (GraphPad Prism 6). In addition, Spearman's r correlation coefficients were calculated to determine the Test–Retest repeatability of the responses provoked by the dual exercise intervention. One subject was excluded because of non-compliance with the instructions with regard to dietary guidelines. Some individuals were excluded from analysis when there were missing blood samples. Therefore, the number of subjects included in each of the analyses is 11, unless stated otherwise in the figure legends. Statistical significance was defined as a two-tailed P < 0.05.

Results

Markers of workload

The impact of the exercise protocol on general metabolic markers, including glucose and lactate, was similar for both Test and Retest (see **Figure 3.2**). This is supported by the similar workload during the glycogen depletion phase and endurance cycling phase of the intervention protocol between Test and Retest (P > 0.05, data not shown). Plasma glucose concentrations were reduced directly after exercise, which was still the case 1 h later. For both Test and Retest, plasma lactate concentrations increased slightly, but significantly, following exercise. These levels returned to resting values within 1 h. Also, serum FFA and BHB concentrations showed similar patterns during rest and exercise, with a significant increase in concentrations following exercise. In contrast to BHB, serum FFA concentrations declined 1 h following exercise, although they were still slightly higher than the rest levels during the Test period (P = 0.03). Each of the metabolic markers showed a significant correlation between the Test and Retest outcomes (**Table 3.2**). There was no significant difference in RER between Test and Retest (**Figure 3.3A**). Indirect calorimetry indicated a decrease in the RER after exercise, implying that the metabolism had shifted towards lipid oxidation, rather than carbohydrate oxidation, after completing the dual exercise protocol (**Figure 3.3B**). There was also no significant difference between Test and Retest in energy expenditure (**Figure 3.3C**). Energy expenditure patterns showed a significant increase in energy expenditure following exercise (**Figure 3.3D**). Intake of casein induced a diet-induced thermogenic effect, as shown by a significant increase (P < 0.001) in the energy expenditure.
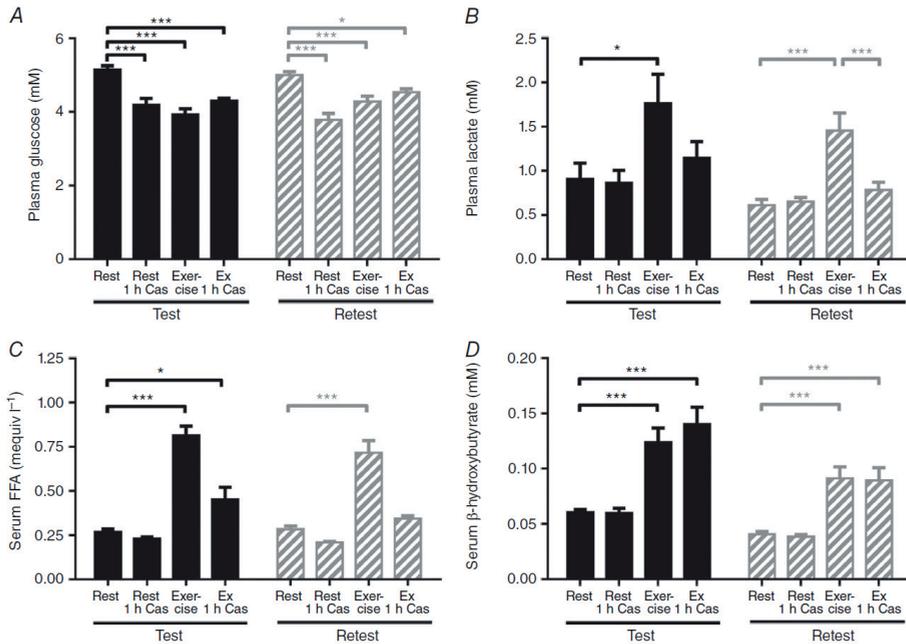
Figure 3.2. The effect of strenuous exercise on markers of energy metabolism

Plasma glucose (A), plasma lactate (B), serum free fatty acids (FFA; C) and serum β-hydroxybutyrate (BHB; D) are shown as mean values + SEM. Comparisons are made with the rest values during the Test (black bars, n = 10 for FFA and BHB) and Retest (grey hatched bars, n = 11) of the experimental protocol. Abbreviation: Cas, casein protein intake. ∗P < 0.05 and ∗∗∗P < 0.001.

Table 3.2. Spearman correlation coefficients to Test – Retest correlation

Parameter	Rest and exercise values		Exercise values	
	Spearman's r correlation coefficient	P-Value	Spearman's r correlation coefficient	P-Value
Glucose	0.580	<0.0001	0.447	0.04
Lactate	0.584	<0.0001	0.809	<0.0001
Free fatty acids	0.811	<0.0001	0.726	0.0002
β-Hydroxybutyrate	0.779	<0.0001	0.583	0.006
Creatine kinase (muscle)	0.074	0.68	−0.332	0.13
Lactulose-to-rhamnose ratio	0.150	0.53	−0.149	0.68
Fibroblast growth factor-21	−0.069	0.67	0.096	0.68
Fractalkine (CX$_3$CL1)	0.596	0.0003	0.444	0.04
Interleukin-6	0.781	<0.0001	0.816	<0.0001
Interleukin-10	0.584	0.0004	0.422	0.05
Interferon-γ inducible protein-10	0.239	0.180	0.215	0.34

Spearman's r correlation coefficient was calculated because values were not normally distributed.
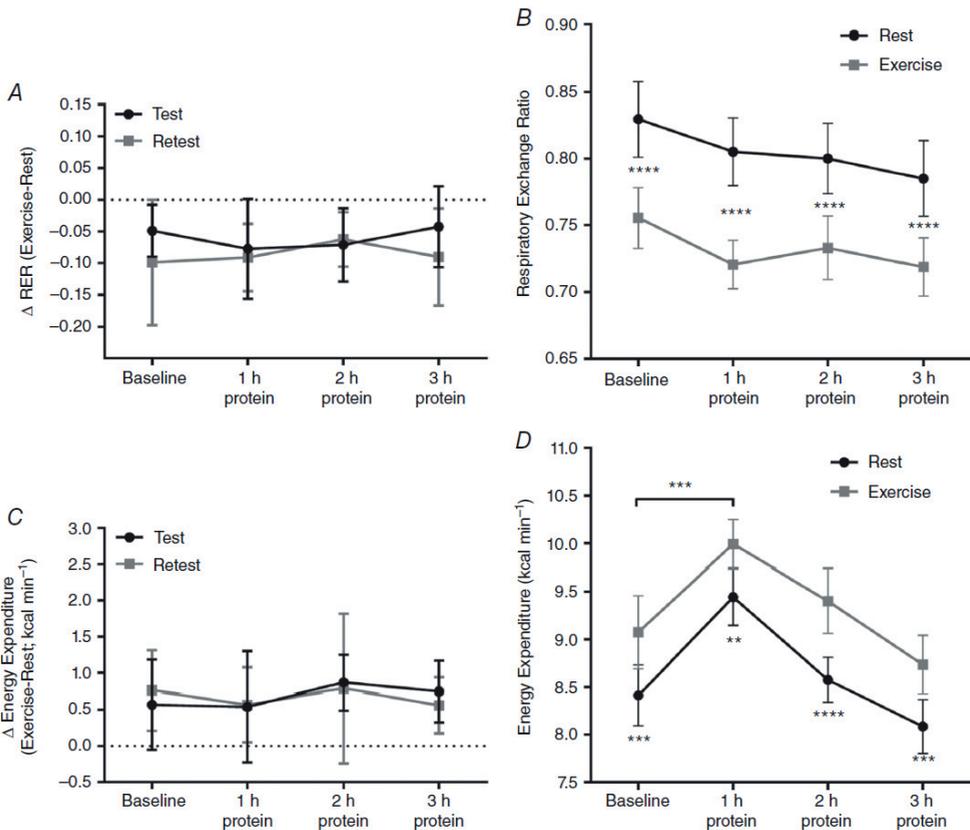
Figure 3.3. The effect of strenuous exercise and casein bolus intake on energy expenditure

The respiratory exchange ratio (RER; A) and energy expenditure (C) are shown as the difference (Δ) between exercise and rest values ± SD (n = 8) at time t = 0 and 1, 2 and 3 h after intake of the sugar test solution and protein shake. To compare rest with exercise, RER values (B) and energy expenditure levels (D) of Test and Retest are taken together (n = 16). ∗∗P < 0.01, ∗∗∗P < 0.001 and ∗∗∗∗P < 0.0001.

Urine metabolomics

Principal component analysis of the urine metabolic profiles revealed a clear separation between the urine metabolite profiles in the rest and exercise conditions (**Figure 3.4**; light and dark blue versus red and green). These shifts were similar for both Test and Retest, and there was no clear difference in metabolite profiles between Test and Retest.
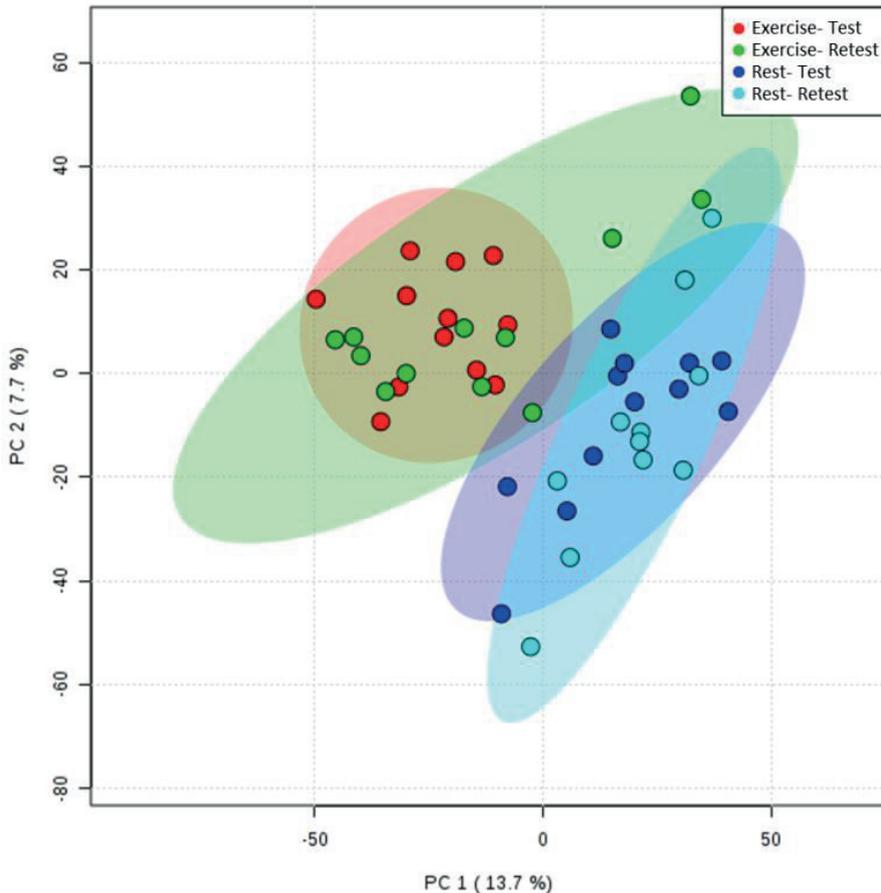
Figure 3.4. Principal component (PC) analysis plot of untargeted urine metabolomics
The principal component analysis of urine metabolomics is shown, comparing the rest and exercise conditions (n = 11 for each condition) for both Test and Retest.

Exercise-induced stress-related markers

In contrast to the metabolic markers, parameters aimed at measuring exercise-induced stress or damage showed different patterns for Test and Retest. To assess exercise-induced effects on intestinal permeability, plasma concentrations of lactulose and L-rhamnose were determined and calculated as the L/R ratios. **Figure 3.5** shows that during the Test period the plasma L/R ratio increased after exercise (P = 0.03). During the Retest period, such an

increase was not observed. Serum concentrations of CKM increased significantly straight after completion of the exercise protocol during the Test period, whereas during the Retest period it took 24 h for the concentrations to increase significantly from resting levels. The recently discovered metabolic marker FGF21 also showed a significant increase in serum concentrations 1 h after exercise, but during the Test period only. For each of the parameters of exercise-induced stress or damage, the effect size was found to be significantly larger during the Test period compared with the Retest period. In line with this finding, Test and Retest values were not significantly correlated with each other (**Table 3.2**).

Exercise-induced cytokine response

For most of the cytokines that were measured, no significant responses were observed (**Table 3.3**). Only four of the measured cytokines showed differences in their concentrations following exercise compared with rest. These cytokines are also shown in **Figure 3.6**. Serum IL-6 and IL-10 concentrations increased after exercise during the Test period only. Serum IP-10 concentrations also only differed during the Test period, with a significant decrease after exercise. Fractalkine was the only exception and showed increased plasma concentrations following exercise compared with rest for both Test and Retest. In contrast to the exercise-induced muscle-related markers, the cytokine responses during Test were significantly correlated with those during the Retest (**Table 3.2**).
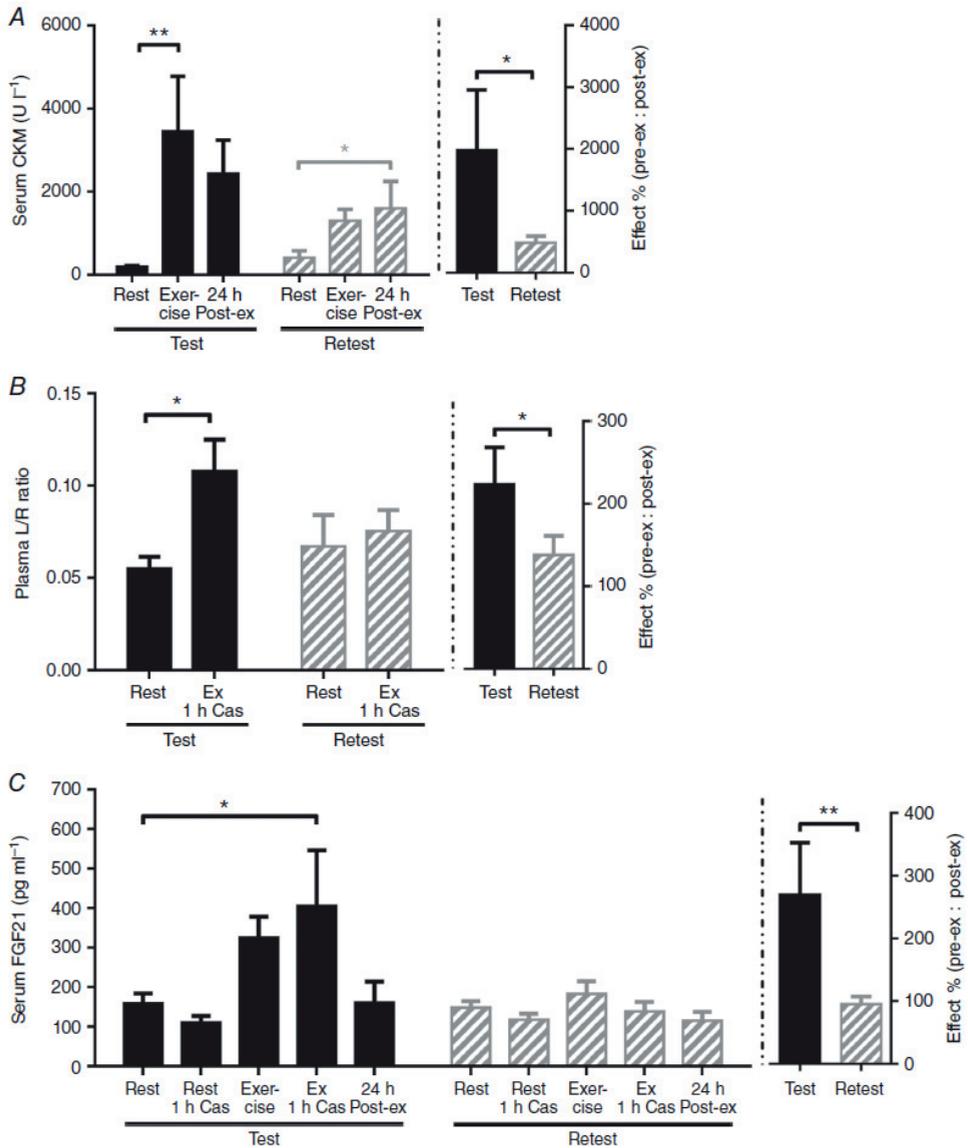
Figure 3.5. The effect of strenuous exercise on intestinal permeability and muscle-stress markers

Levels of serum muscle-specific creatine kinase (CKM; A), plasma lactulose-to-rhamnose (L/R) ratio (B) and serum fibroblast growth factor 21 (FGF21; C) are shown as mean values + SEM. Comparisons are made with the rest values during execution of the Test (black bars, n = 10) and Retest protocols (grey hatched bars, n = 11). The effect percentage is calculated for each individual. For serum CKM, the rest value is compared with the value postexercise (Post-ex); for the plasma L/R ratio and the serum FGF21 concentrations, the rest value is compared with the value 1 h after exercise. Abbreviation: Cas, casein protein intake. $*P < 0.05$ and $**P < 0.01$.

Table 3.3. Exercise-induced cytokine responses during two protocol executions

Parameter	LLOQ (pg ml⁻¹)	Test						Retest					
		Rest		Exercise		Exercise 1 h Casein		Rest		Exercise		Exercise 1 h Casein	
		Average (pg ml⁻¹)	SD	Average (pg ml⁻¹)	SD	Average (pg ml⁻¹)	SD	Average (pg ml⁻¹)	SD	Average (pg ml⁻¹)	SD	Average (pg ml⁻¹)	SD
Fractalkine (CX₃CL1)	156.25	545.8	64.25	675.9*	133.3	812.1*	158.1	593.6	119.2	737.9*	147.9	799.6	225.7
IL-6	2.29	3.65	3.40	9.03*	6.47	8.40	7.94	6.93	10.85	5.68	4.40	5.16	4.10
IL-10	2.42	1.34	0.87	15.73	10.50	29.85*	38.15	2.12	1.49	4.96	3.30	5.78	5.08
IP-10 (CXCL10)	1.00	260.45	70.18	190.90*	37.97	190.52*	35.07	399.10	335.15	304.70	235.60	301.43	253.52
IL-1RA	29.34	92.32	250.77	126.67	235.08	498.12	884.95	101.02	212.96	106.64	233.29	119.28	211.07
IL-1α	1.47	11.14	25.27	9.47	21.29	11.74	22.71	10.46	20.30	10.64	21.64	9.00	18.38
IL-1β	1.14	0.49	1.15	0.54	0.97	0.56	1.01	0.38	0.66	0.40	0.71	0.29	0.63
IL-2	2.34	0.85	1.12	1.15	1.19	1.57	1.93	0.88	1.20	1.01	1.27	1.07	1.16
IL-5	1.64	25.62	60.37	26.08	55.63	28.51	57.62	24.22	46.90	24.27	50.64	21.51	45.92
IL-12	5.97	2.95	6.52	3.20	5.15	3.46	5.11	2.99	4.03	2.85	4.61	1.82	2.49
IL-13	1.23	74.26	172.12	72.35	152.13	81.31	159.81	67.32	133.39	72.27	143.83	65.24	132.15
IL-25	80.45	599.79	1028.86	565.05	894.23	678.97	1010.75	637.75	903.71	635.01	995.07	572.03	842.85
TNF-α	1.21	3.92	7.89	3.50	7.54	3.45	7.32	3.74	6.95	3.48	7.20	3.15	6.35
IFN-γ	3.11	17.13	39.38	18.35	37.86	18.99	37.99	17.22	34.42	17.03	34.62	15.38	31.27
LIGHT	30.96	120.16	212.39	117.01	202.54	140.12	215.02	124.46	208.57	116.83	208.11	108.52	186.24
TSLP	0.30	7.78	18.25	6.38	15.68	8.36	17.01	6.87	13.83	7.28	14.65	6.69	13.85
G-CSF	9.48	173.46	342.66	178.48	324.65	179.75	329.25	173.16	311.45	175.60	316.75	154.63	271.08

Comparisons are made with the values found in the rest condition (rest). *$P < 0.05$. The lower limit of quantification (LLOQ) was defined as the lowest measured level in the current assay. Abbreviations: IL, interleukin; IP-10, IFN-γ- induced protein 10; RA, receptor antagonist; TNF-α, tumor necrosis factor alpha; IFN-γ, interferron gamma; LIGHT, tumor necrosis factor superfamily member 14; TSLP, thymic stromal lymphopoietin; G-CSF, granulocyte-colony stimulating factor.
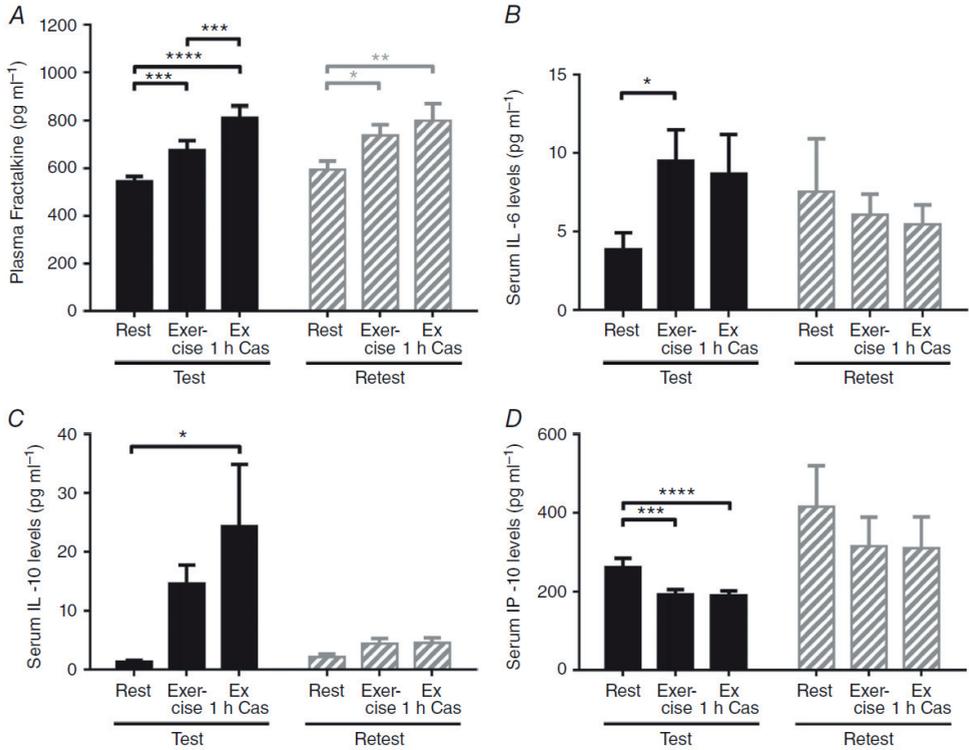
Figure 3.6. The effect of strenuous exercise on cytokine response
Plasma concentration of fractalkine (A), serum concentration of interleukin (IL)-6 (B), serum concentration of IL-10 (C) and serum IP-10 (D) are shown as mean values + SEM. Comparisons are made with the rest values during the Test (black bars, n = 11) and Retest (grey hatched bars, n = 11). Abbreviation: Cas, casein protein intake; IL, interleukin; IP-10, interferon gamma-induced protein 10. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, and $****\ P < 0.0001$

Discussion

The aim of the present study was to investigate test–retest repeatability of exercise-induced stress responses in well-trained subjects. Therefore, we measured a set of parameters for energy metabolism, markers of metabolic or cellular stress, and immunological factors. The finding that parameters for general energy metabolism (glucose, lactate, FFA and BHB) and energy expenditure did not differ between the Test and Retest period indicates that the subjects delivered a similar workload. Remarkably, most immune markers were found not to be affected by exercise in these well-trained individuals. The effects of the exercise protocol on systemic concentrations of CKM and FGF-21, the L/R ratio and concentration of some of the myokines, however, showed distinct patterns between the Test and Retest periods. The increase in concentrations of CKM, FGF-21, IL-6 and IL-10 and the increase in the L/R ratio were diminished or even absent during the Retest period compared with the Test period. These results suggest adaptation when the exercise protocol is repeated, even in these well-trained individuals. The adaptation in intestinal permeability as measured with the L/R ratio suggests that the gut may be, at least to some extent, 'trainable', which could result in a reduction of the often reported gastrointestinal complaints by endurance athletes [22]. A clear distinction was observed between the urine metabolic profiles before and after exercise, but not between the Test and Retest. These results suggest that metabolites reflecting metabolic stress or tissue damage, although expected to be present, do not dominate the differences in metabolic profiles between rest and exercise. When two bouts of exercise are performed within a short time period (e.g. within 24 h), a carry-over effect in exercise-induced responses is often seen [23-25]. In the present study, however, the opposite was seen with the responses during the Retest compared with the Test, with the exception of IL-6. One week of washout, as applied in the present study, is quite long. There might be, however, a carry-over-like effect of the defence mechanisms activated during the test period that are still active during the Retest, thereby attenuating the stress response [26]. The considerable interindividual variation found in metabolic stress and tissue damage responses to the exercise protocol, especially during the Test period, may also, at least in part, be explained by this possible carry-over effect.

The response levels of the myokine fractalkine were similar for the Test and Retest periods. This finding supports earlier results by Catoire & Kersten [11], who designated fractalkine as an 'exercise factor' which, according to their definition, should not adapt to training. Interleukin-10, in contrast, seems to be responsive to adaptation to repeated exercise and could be considered an adaptive exercise factor. In contrast, the possible carry-over effect of the Test protocol to the Retest protocol on the concentrations of IL-6 suggests that excluding intense physical activity for 3 days prior to a study trial may not be long enough to prevent carry-over effects from prior exercise. It is important to distinguish the effects that are attributable to adaptation from those resulting from carry-over for functional interpretation of outcomes of exercise protocols.

For some exercise-induced stress markers, such as the newly discovered marker FGF21, effects of exercise protocols were inconsistent in earlier studies, leading to disagreement on the relationship between exercise and FGF21 [9-12]. This can, at least in part, be attributable to the large differences between the exercise protocols, ranging from a single bout of exercise to several weeks of exercise intervention. However, response adaptation, as shown in the present study, could also form part of the basis of these inconsistent findings. It is interesting to speculate on the functional role of a stress response and subsequent adaptation by, for example, FGF21 induction. Strenuous exercise is associated with endoplasmic reticulum stress, which can result in skeletal muscle damage at excessive levels [27]. Secretion of FGF21 from skeletal muscle is induced in response to skeletal muscle endoplasmic reticulum stress leading to the activation of the integrated stress response, labelling FGF21 as a muscle-related stress marker rather than an exercise-induced myokine [27]. A transient elevation in endoplasmic reticulum stress is thought to be necessary for adaptive improvement through mitohormesis [27]. Repeated exercise could result in a higher resistance against skeletal muscle endoplasmic reticulum stress and, subsequently, also in an attenuated rescue signal of FGF21 [28].

Conclusions

There seems to be an adaptation to exercise-induced stress or damage responses in well-trained healthy young men. Perhaps this is attributable to a carry-over effect of the defence mechanisms triggered during the Test. This finding may explain, in part, inconsistent outcomes in the literature concerning several factors of exercise-induced stress. It also has implications for the design of protocols to assess exercise-induced responses. More research is required to determine the washout period needed to prevent adaptation and carry-over effects of prior exercises. When exercise interventions are not repeated within the study design, the duration of exercise restrictions prior to the start of the study may be an especially important factor to take into account.

Additional information

Competing interests

Author contributions

the paper and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

References

1. Bischoff, S.C., et al., Intestinal permeability – a new target for disease prevention and therapy. BMC Gastroenterology, 2014. 14(1): p. 189.

2. Quigley, E.M.M., Leaky gut - concept or clinical entity? Current Opinion in Gastroenterology, 2016. 32(2): p. 74-79.

3. Øktedalen, O., et al., Changes in the gastrointestinal mucosa after long-distance running. Scandinavian Journal of Gastroenterology, 1992. 27(4): p. 270-274.

4. Pals, K.L., et al., Effect of running intensity on intestinal permeability. Journal of Applied Physiology, 1997. 82(2): p. 571-576.

5. Ryan, A.J., R. Chang, and C.V. Gisolfi, Gastrointestinal permeability following aspirin intake and prolonged running. Medicine and Science in Sports and Exercise, 1996. 28(6): p. 698-705.

6. Marchbank, T., et al., The nutriceutical bovine colostrum truncates the increase in gut permeability caused by heavy exercise in athletes. American Journal of Physiology - Gastrointestinal and Liver Physiology, 2011. 300(3): p. G477-G484.

7. Van Nieuwenhoven, M.A., F. Brouns, and R.J. Brummer, The effect of physical exercise on parameters of gastrointestinal function. Neurogastroenterology & Motility, 1999. 11(6): p. 431-439.

8. Van Wijck, K., et al., Exercise-induced splanchnic hypoperfusion results in gut dysfunction in healthy men. PLoS ONE, 2011. 6(7): p. e22366.

9. Kim, K.H., et al., Acute exercise induces FGF21 expression in mice and in healthy humans. PLoS ONE, 2013. 8(5): p. e63517.

10. Cuevas-Ramos, D., et al., Exercise Increases Serum Fibroblast Growth Factor 21 (FGF21) Levels. PLoS ONE, 2012. 7(5): p. e38022.

11. Catoire, M. and S. Kersten, The search for exercise factors in humans. The FASEB Journal, 2015. 29(5): p. 1615-1628.

12. Yang, S.J., et al., Effects of a three-month combined exercise programme on fibroblast growth factor 21 and fetuin-A levels and arterial stiffness in obese women. Clinical Endocrinology, 2011. 75(4): p. 464-469.

13. Bassett, D. and E.T. Howley, Limiting factors for maximum oxygen uptake and determinants of endurance performance. Medicine and Science in Sports and Exercise, 2000. 32(1): p. 70-84.

14. Hubal, M.J., et al., Variability in muscle size and strength gain after unilateral resistance training. Medicine and Science in Sports and Exercise, 2005. 37(6): p. 964-72.

15. Carol, A., et al., Bovine Colostrum Supplementation's Lack of Effect on Immune Variables During Short-Term Intense Exercise in Well-Trained Athletes. International Journal of Sport Nutrition and Exercise Metabolism, 2011. 21(2): p. 135.

16. Tautenhahn, R., C. Böttcher, and S. Neumann, Highly sensitive feature detection for high resolution LC/MS. BMC Bioinformatics, 2008. 9(1): p. 1.

17. Xia, J., et al., MetaboAnalyst 3.0—making metabolomics more meaningful. Nucleic Acids Research, 2015. 43(W1): p. W251-W257.

18. Van Nieuwenhoven, M., et al., The sensitivity of the lactulose/rhamnose gut permeability test. European Journal of Clinical Investigation, 1999. 29(2): p. 160-165.

19. Haase, A.M., et al., Dual sugar permeability testing in diarrheal disease. The Journal of Pediatrics, 2000. 136(2): p. 232-237.

20. van Wijck, K., et al., Novel analytical approach to a multi-sugar whole gut permeability assay. Journal of Chromatography B, 2011. 879(26): p. 2794-2801.

21. de Jager, W., et al., Improved multiplex immunoassay performance in human plasma and synovial fluid following removal of interfering heterophilic antibodies. Journal of Immunological Methods, 2005. 300(1–2): p. 124-135.

22. de Oliveira, E.P., R.C. Burini, and A. Jeukendrup, Gastrointestinal Complaints During Exercise: Prevalence, Etiology, and Nutritional Recommendations. Sports Medicine, 2014. 44(1): p. 79-85.

23. Ronsen, O., et al., Leukocyte counts and lymphocyte responsiveness associated with repeated bouts of strenuous endurance exercise. Journal of Applied Physiology, 2001. 91(1): p. 425-434.
24. McFarlin, B., et al., Repeated endurance exercise affects leukocyte number but not NK cell activity. Medicine and Science in Sports and Exercise, 2003. 35(7): p. 1130-1138.
25. Robson-Ansley, P., et al., The effect of repeated endurance exercise on IL-6 and sIL-6R and their relationship with sensations of fatigue at rest. Cytokine, 2009. 45(2): p. 111-116.
26. Shing, C.M., et al., The effect of consecutive days of exercise on markers of oxidative stress. Applied Physiology, Nutrition, and Metabolism, 2007. 32(4): p. 677-685.
27. Ost, M., et al., Regulation of myokine expression: Role of exercise and cellular stress. Free Radical Biology and Medicine, 2016.
28. Ahn, B., et al., Diaphragm atrophy and contractile dysfunction in a murine model of pulmonary hypertension. PLoS ONE, 2013. 8(4): p. e62702.

Chapter 4

Detection of peanut allergen in human blood after consumption of peanuts is skewed by endogenous immunoglobulins

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Highlights

- Peanut allergen can be detected with ELISA in serum after peanut consumption
- Ara h 6-specific IgG and IgG4 antibodies inhibit detection of Ara h 6 in serum
- This inhibition may be a broader phenomenon in studies on uptake of food allergens
- This may explain variable serum levels of food allergen reported in literature

Abstract

Some studies have suggested that allergens may appear in the circulation after ingestion of allergenic food sources. The reported levels of allergen in serum, however, are low, and conclusions between studies differ. Here, we investigated factors that determine the detection of allergens in serum after consumption of peanuts.

Ten healthy volunteers ingested 100 g of light-roasted peanuts. Serum samples were taken at regular intervals for six hours. A double monoclonal sandwich ELISA was used to analyse the presence and quantity of the major peanut allergen Ara h 6 in serum.

In 4 out of 10 subjects, no Ara h 6 could be detected. Purified Ara h 6 that was digested *in vitro* was still reactive in the ELISA, rejecting the possibility that digestion leads to small peptides that could not be detected. Spiking of purified Ara h 6 in baseline serum showed that the pre-ingestion serum of these four subjects partially prevented Ara h 6 to react in the ELISA, with a reduction of reactivity of up to 3 orders of magnitude or more. Pre-ingestion serum of the other six subjects did not show such an effect. The reduction of reactivity of Ara h 6 coincided with high titres of IgG and IgG4, and removal of IgG from pre-ingestion serum abolished this effect completely, indicating that IgG and IgG4 inhibited the reactivity of Ara h6 in the ELISA.

We conclude that some individuals have IgG and IgG4 against food allergens in their blood, which interferes with detection of such food allergens in serum. Because this effect does not occur for each individual, the possibility of such interference should be taken into consideration when interpreting immunochemical studies on the absorption of food allergens in serum.

Introduction

Food allergies affect 2–3% of the population in Western countries, and peanut allergy is one of the most important food allergies because of 1) its persistent character, 2) the low amounts of peanut needed to trigger a reaction, and 3) peanut being a widely used food ingredient. Peanut conglutins Ara h 2 and Ara h 6 are the most potent allergens with regard to IgE-binding, skin prick test, and basophil test. IgE to these allergens has a high positive predictive value for diagnosing peanut allergy [1].

A characteristic of conglutins (2S–albumins) is their relative stability to digestion [2, 3]. Digestion of Ara h 2 and Ara h 6 by pepsin and trypsin has been investigated in the past, showing that these are only partially hydrolysed resulting in relatively large peptides of around 4 to 5 kDa and 10 kDa in size [4-6]. Such digestion-resistant peptides are exposed to the intestinal immune system as large protein fragments, potentially explaining the potent allergenicity of these allergens with regard to their capacity to sensitize. It has also been shown that digestion-resistant peptides can be taken up in the circulation, leading to systemic reactions in peanut allergic individuals. Several studies investigated the uptake of digestion-stable food allergens such as peanut [7, 8], egg [9] and wheat [10] in healthy individuals and animal models [11]. Ara h 2 and Ara h 6 have also been detected in breast milk of lactating women [12, 13] and mice [11]. Moreover, a case report revealed that transfusion of a blood product into a peanut allergic recipient led to an anaphylactic reaction explained by residual peanut in the blood of one of the donors who consumed peanut prior to donation [14]. Together these reports indicate that immunologically active fragments of peanut allergens can at least partially survive the gastro-intestinal tract, and may appear in the circulation to be transported to various compartments of the body. However, the reported values of peanut allergen taken up are low, and often close to the limit of detection. Also, large variability between studies and between individuals within studies is observed. Considering the digestion resistance of peanut conglutins and their fairly high abundance in peanut, one would expect more consistent and higher levels of such proteins in the circulation if these were indeed taken up by the intestine and transported into the blood.

The aim of this work was to identify possible confounding factors for the detection of peanut allergens in serum after consumption of peanut. We selected Ara h 6 as representative of the potent peanut conglutin allergens. Ara h 6 is biochemically well characterised and appears as a homogenous protein, with limited isoforms. We recruited a group of 10 healthy individuals, sampled blood before and after digestion of peanuts and analysed their serum for the presence of Ara h 6 at various time points. Moreover, we performed spiking experiments to investigate if detected levels corresponded to expected levels, and which sera-derived factors could be responsible for interference.

Materials and Methods

Healthy volunteers / subject

Ten healthy subjects were recruited via social media and email database of the university's human nutrition division (Wageningen University, the Netherlands). Exclusion criteria were having (suspected) allergic symptoms after peanut ingestion, smoking, use of illicit drugs, NSAIDs on a chronic basis, or use of any medication for gastric or intestinal complaints. None of the subjects donated blood during the last six weeks before the start of the study. Subjects were also instructed not to eat peanut-containing foods nor use alcohol for 2 days prior to the test day.

This study was approved by the medical ethical committee of Wageningen University (METC-WU 15/34; NTR5655), and conducted in accordance with the Declaration of Helsinki (revised version, October 2008, Seoul). All subjects gave their written informed consent after the nature and possible consequences of the study had been fully explained.

Each subject came to the university while having fasted overnight. Pre-ingestion blood was sampled after which they were asked to consume 100 g of light-roasted, unsalted peanuts obtained from a local supermarket (Albert Heijn; private label product) within 10 min blood was sampled in serum separator tubes 30, 60, 120, 240, and 360 min after finishing peanut consumption. Blood was left to clot in the dark for at least 20 min at room temperature, after which the tubes were centrifuged at 2000 g for 10 min at room temperature to obtain serum, which was directly aliquoted and stored at − 80 °C until further analysis.

Peanut allergens

Virginia peanuts obtained from the US were used to purify the various Ara h 6 preparations. Ara h 6 was purified essentially as described earlier [15] with one additional hydrophobic interaction chromatography step to remove traces of impurities. Post-translationally proteolytically processed Ara h 6 (hereafter named naturally processed Ara h 6) was purified from side fractions of the Ara h 6 purification (Koppelman S.J., unpublished data), which contained disulfide-linked molecules consisting of a 5 and 10 kDa chain with estimated purify of > 95%. Identity was proven by peptide mass finger printing (data not shown). Digestion-resistant peptides from peanut conglutin (equimolar mix of the heavy isoform of Ara h 2, the light isoform of Ara h 2, and Ara h 6 were prepared by incubation of peanut conglutin with immobilized trypsin [6]. The fraction of Ara h 6 in the equimolar mix was 0.30 based on mass. The protein concentration of the digestion-resistant peptide mix (hereafter named digested mix of Ara h 2 and Ara h 6) is expressed in mg of Ara h 6 per mL to simplify comparison with other Ara h 6 preparations.

ELISAs

The Ara h 6 ELISA was from Indoor Biotechnologies (EL-AH 6, Cardiff, UK), utilizing two monoclonal antibodies. The instructions of the manufacturer were followed, except that in some cases the reference material was replaced by other Ara h 6 preparations. By using

extra dilutions of the standard, the lower limit of quantification was determined to be 0.024 ng/mL (defined as the value corresponding to an absorbance of the blank + 10 SD), and the lower limit of detection to be 0.012 (defined as the value corresponding to an absorbance of the blank + 3 SD). Samples were tested in duplicate and experiments were performed at least twice. To compare reactivity of different Ara h 6 forms, EC50 values, defined as the concentration that leads to half-maximal absorbance, were calculated using the Graphpad Prism software, version 6.07.

ELISAs for IgG and IgG4 were set-up as follows. Purified Ara h 6 was coated on ELISA plates (Nunc MaxiSorp® flat-bottom 96 well plate) at 3 µg/mL in PBS (pH 7.4; 136.89 mM NaCl, 1.47 mM KH_2PO_4, 8.10 mM Na_2HPO_4, 2.68 mM KCl), 100 µL/well, overnight at 4 °C. Plates were washed once with PBS and then blocked with PBS containing 2% (W/W) bovine serum albumin (BSA, A6003, Sigma Aldrich, Zwijndrecht, NL), for 2 h at 37 °C under shaking conditions (300 rpm). After washing (3 times with PBS containing 0.05% Tween 20 (PBS-T)), diluted serum samples were pipetted in the wells in duplicate and were allowed to incubate for 2 h at 37 °C under shaking conditions (300 rpm). Wells were washed again (3 times with PBS-T) and a 1:500 dilution of horseradish peroxidase conjugated anti-human IgG (05–4220, Invitrogen, Breda, NL) or 1:500 dilution of anti-human IgG4 (ab99817, Abcam, Cambridge, UK) in PBS-T with 1% BSA was applied on the plates for 1.5 h at 37 °C under shaking conditions (300 rpm). Plates were further developed using 100 µL of TMB substrate (T0440, Sigma Aldrich). Colour development was stopped after 10 min by adding 100 µL of Stop reagent (S5814, Sigma Aldrich). Absorbances with oversaturated values were imputed at 4.1, representing the highest absorbance that can be measured with the ELISA plate reader (Synergy HT, BioTek, Winoski, VT, USA).

Removal of IgG from serum

Two times diluted pre-digestion serum (in PBS) was applied to a Protein G Sepharose Column (6518, BioVision, ITK Diagnostics bv, Uithoorn, NL), equilibrated with PBS. The flow-through was collected and re-applied to the column four times. The final flow-through was collected as IgG-depleted serum. The column was washed and re-equilibrated according to the instructions of the manufacturer.

Results and Discussion

We applied an ELISA specific for Ara h 6 detection to study the presence of this allergen in serum of healthy individuals after consuming peanuts. Several hypotheses were tested to explain why the detected levels of Ara h 6 are low and variable.

Hypotheses formulation

An overview of some relevant subject characteristics of the study is provided in **Table 4.1**. Upon ingestion of peanuts, the serum samples at t = 60 min were tested for the presence and quantity of Ara h 6. In serum of four subjects (out of ten), Ara h 6 was undetectable,

while in the serum of the other six subjects low levels of Ara h 6 of 0.30 ± 0.16 ng/mL could be detected. The total amount of peanuts consumed was 100 g. Using a protein content of 25% [16], and taking into account that the Ara h 6 content of (Virginia) peanuts is approximately 6% [17], the subjects ingested approximately 1.5 g of Ara h 6. If all had been taken up in the circulation, assuming no turnover of Ara h 6 and a blood volume of 5 L, this would have led to a concentration of 0.3 g/L, corresponding to 300,000 ng/mL. It is expected that the intestine functions as a barrier, preventing the uptake of larger undigested dietary components. The observation, however, that only 0.3 ng/mL is found, and not even in serum from all subjects, possibly illustrates a minor and variable part of ingested Ara h 6 being detected in serum. Three hypotheses were formulated to explain the low levels of uptake of Ara h 6 in serum. In these hypotheses we assume that there are additional factors beyond intestinal uptake function: 1) the ELISA is not reactive for hydrolysed or digested Ara h 6; 2) a matrix effect disturbs the analytical procedure; or 3) the time point chosen at first for serum collection of 60 min is not optimal. The sections below provide the results of testing the hypotheses.

Table 4.1. Subject characteristics and Ara h 6 levels.

ID	M/F	Age	Consumption products high peanut content[1]	Consumption products low peanut content[2]	[Ara h 6] at 60 min (ng/mL)
1	M	21	Almost daily	1-3× per month	ND
2	F	20	2-5× per week	1-3× per month	0.38
3	F	22	1-3× per month	2-5× per week	0.09
4	M	21	1-3× per month	1× per week	0.15
5	F	21	2-5× per week	1-3× per month	0.26
6	F	22	2-5× per week	<1× per month	0.36
7	F	22	1-3× per month	2-5× per week	0.54
8	M	23	1× per week	1-3× per month	ND
9	M	21	Almost daily	1-3× per month	ND
10	F	23	1-3× per month	1-3× per month	ND

M/F = Male/Female.
ND = Not detected.
[1] Examples: peanuts/peanut butter/ peanut sauce.
[2] Examples: candy bars/ cookies/ muesli (bars).

Reactivity of hydrolysed or digested Ara h 6 in the Ara h 6 ELISA

The epitopes that are recognised by the monoclonal antibodies utilised in the Ara h 6 ELISA are, to our knowledge, not mapped (personal communication with ELISA manufacturer, dd. March 2016). The ELISA manufacturer also does not provide information on the possible reactivity of processed or digested Ara h 6. It is therefore not known whether this ELISA can detect digested Ara h 6. A naturally hydrolysed (processed) form of Ara h 6 occurs naturally in peanut [18] and was recently purified and characterized biochemically (Koppelman S.J., unpublished data). This post-translationally proteolytically processed form is composed of an N-terminal part of about 5 kDa linked by disulphide bonds to the C-terminal part of about 10 kDa, highly similar to the profile for digested Ara h 2 and Ara h 6 [6], and was used to

investigate the detection of digested Ara h 6 by the ELISA. The reactivity of the three different Ara h 6 preparations was tested in the Ara h 6 ELISA. **Figure 4.1** shows the calibration curve of the purified (intact) Ara h 6, a curve of the naturally processed form of Ara h 6, and a curve of a mix of Ara h 2 and Ara h 6 treated with trypsin (normalised for Ara h 6 concentration). Intact and naturally processed Ara h 6 show a comparable reactivity illustrated by EC50 values (concentration that leads to half-maximal absorbance) of 2.6 and 3.0 ng/mL, respectively. To mimic digestion under more physiological conditions, a mix of Ara h 2 and Ara h 6 was treated with trypsin, leading to a protein profile for Ara h 6 consisting of a 5 kDa and 10 kDa fragment held together by disulphide bridges as described above [6]. These digestion-resistant peptides were also tested in the Ara h 6 ELISA, and show a curve overlapping with that of naturally processed Ara h 6 (EC50 is 2.8 ng/mL). Together, this strongly suggests that Ara h 6 digested *in vivo* is still reactive in the Ara h 6 ELISA, although perhaps at a slightly lower level. It is therefore unlikely that digestion of Ara h 6 prevents the detection by ELISA. The processed form of Ara h 6 purified from peanut was used as standard for the following ELISA experiments. The dynamic range of the calibration curve is 3 orders of magnitude, which allows the detection of large differences in reactivity of samples.
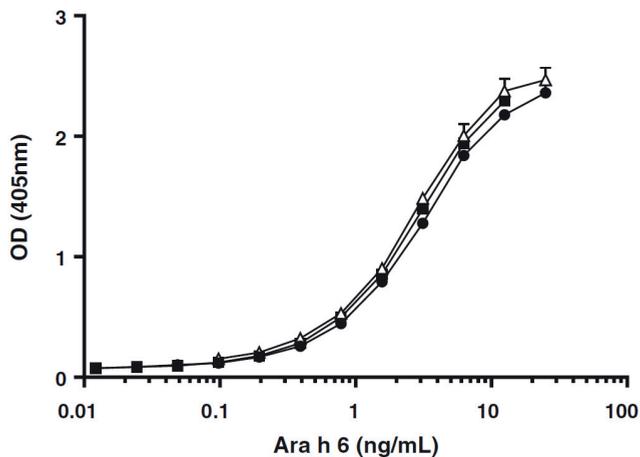
Figure 4.1. Reactivity of Ara h 6 and hydrolysed forms of Ara h 6 in the Ara h 6 ELISA
Open triangles (Δ): Purified native (intact) Ara h 6; Closed circles (●): naturally processed Ara h 6 as purified from peanuts; Closed squares (■): mix of Ara h 2 and Ara h 6 treated with trypsin (Ara h 6 concentration in the mix was calculated and used on X-axis (as described in Methods) to enable comparison with other Ara h 6 forms).

Matrix effects of serum

A serum sample was obtained from all subjects before eating peanuts. This pre-ingestion serum was spiked with various concentrations of naturally processed Ara h 6 to investigate any possible matrix effect. **Figure 4.2** shows that in the majority of sera Ara h 6 could be

detected comparable to Ara h 6 in buffer. However, a marked inhibition of reactivity is observed in sera from four subjects. For subject no. 10, this inhibition is pronounced for low concentrations of Ara h 6, while detection was good for higher concentrations. For subjects no. 1, 8, and 9, the inhibition was much stronger, and for subject no. 9 the detection of even the highest concentration of Ara h 6 (12.5 ng/mL) was still very low. Taking into account the LOD of the ELISA, i.e. 0.012 ng/mL, this inhibitory effect is at least 3 orders of magnitude.

We hypothesized that competing antibodies present in serum may cause the observed inhibitory effect. Immunoglobulin (Ig) toward dietary proteins is common and IgG may increase as a result of frequent ingestion of immune-reactive dietary proteins [19, 20]. A strong increase in IgG antibodies against food proteins such as peanut and egg is seen in immunotherapy studies. Oral immunotherapy with allergic subjects that normally avoid the offending food often results in an increase of serum IgG and functional blocking activity is demonstrated in basophil histamine release assays [21-24]. However, our subjects do not avoid but are regularly exposed to peanut (**Table 4.1**), and it is possible that they have IgG to peanut in their circulation.
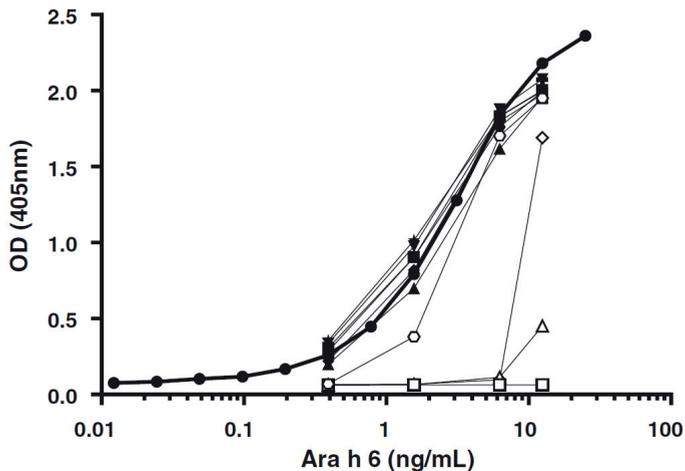
Figure 4.2. Reactivity of naturally processed Ara h 6 when mixed into pre-ingestion serum for the study subjects
Calibration curve (closed circles: ●) is shown for the full dilution range. For spiking the baseline sera 0.4 ng/mL, 1.6 ng/mL, 6.3 ng/mL, and 12.5 ng/mL of naturally processed Ara h 6 was used. Subject no. 1: ◊ ; subject no. 2: ▼ ; subject no. 3: ▲ ; subject no. 4: ■ ; subject no. 5: ; subject no. 6: ♦; subject no. 7: ; subject no. 8: Δ ; subject no. 9: □ ; subject no. 10: .

We have tested the pre-ingestion sera of the subjects in a direct ELISA for Ara h 6-specific IgG (**Figure 4.3A**) and Ara h 6-specific IgG4 (**Figure 4.3B**). The sera that did not show inhibitory effects in the Ara h 6 ELISA had low or absent IgG reactivity. In contrast, the sera from subject no. 1, 8, and 9 had considerable IgG reactivity for Ara h 6. The IgG titres coincided with the inhibitory potency. Interestingly, this picture is even more pronounced

for antibodies of the IgG4 subclass directed to Ara h 6 (**Figure 4.3B**). Serum from subject no. 8 and 9 could be diluted over 1000 fold before losing reactivity. The sera that did not show an inhibitory effect in the Ara h 6 ELISA did not show clear IgG4 reactivity. Further evidence that IgG/IgG4 mediate the inhibitory effect is obtained when IgG was removed from serum using Protein G Sepharose. **Figure 4.3C** shows that IgG and IgG4 was removed efficiently (using the sera with highest titres; i.e. from subjects no. 8 and no. 9) and this resulted in almost complete abolishment of the inhibitory effect on Ara h 6 detection (**Figure 4.3D**). While in untreated serum from subject no. 8 and no. 9 a serum-dilution dependent effect on detection is observed, the IgG-depleted sera allowed detection and quantification of the Ara h 6 spikes well (> 80% at a serum dilution of 4-fold; **Figure 4.3D**).

A large inhibitory capacity of the serum coincided with a high titre of Ara h 6-specific IgG and IgG4. High IgG and IgG4 levels are seen especially in subjects with frequent habitual peanut ingestion, suggesting a possible association. For both characteristics of the sera the order of the effect was: subject no.9 > no.8 > no.1 > no.10. Moreover, the sera from subjects that showed no inhibitory effect also had low or non-detectable Ara h 6-specific IgG or IgG4 titres. Thus, we conclude that for part of the subjects there is an inhibitory effect mediated by IgG and/or IgG4 against Ara h 6 that interferes with and skews the quantification of Ara h 6 by ELISA. Earlier studies reported interaction of food proteins with immunoglobulins, in particular observed in patients with Coeliac disease [25, 26]. However, a potential inhibiting effect of such interaction on the detection of food allergens by immunochemical methods has not been considered so far.
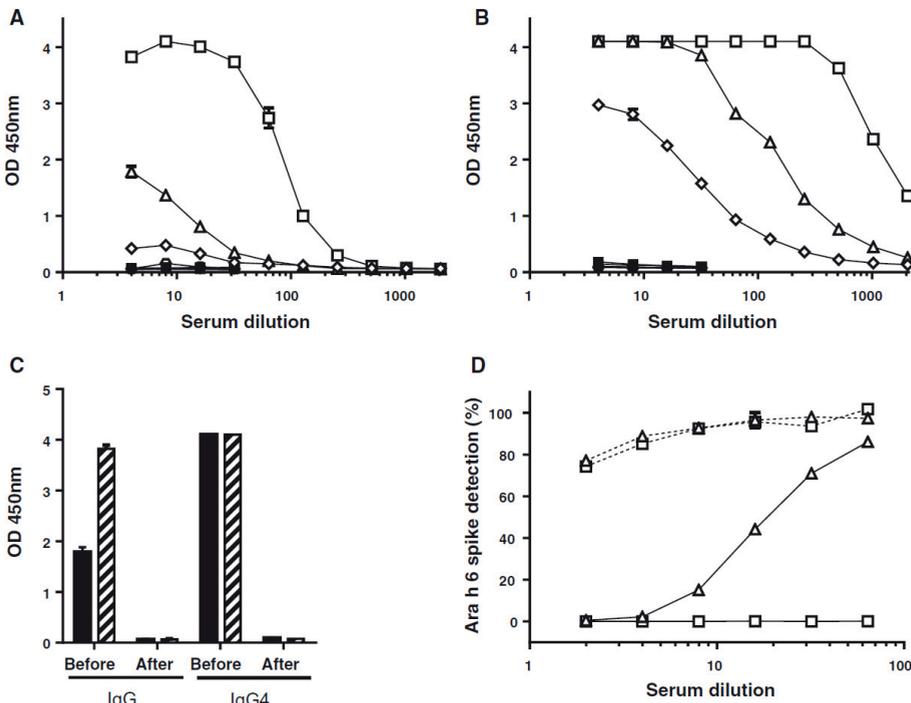
Figure 4.3. IgG and IgG4 specific for naturally processed Ara h 6 in serum of study subjects inhibits reactivity in the Ara h 6 ELISA

IgG and IgG4 ELISAs were performed to compare the Ara h 6-specific IgG and IgG4 titers of the sera from the study subjects. Markers are the same as in Figure 4.2. Panel A: Ara h 6-IgG titers of all subjects. Panel B: Ara h 6-sepecific IgG4 titers for all subjects. Panel C: Ara h 6-specific IgG and IgG4 in serum from subjects no. 8 (black bar) and 9 (Striped bar) (subjects with highest inhibitory potency) before and after cleanup with Protein G Sepharose. Panel D: Detection of different levels of naturally processed Ara h 6 in serum from subject no. 8 and 9 (subjects with lowest detection of naturally processed Ara h 6 spike) before and after cleanup with Protein G Sepharose. Solid line: befoe cleanup; dotted line: after cleanup.

Time course of appearance of Ara h 6 in serum

Due to the inhibitory effects of serum of subject no. 1, 8, 9, and 10 it was not possible to assess the amount of Ara h 6 that enters the circulation of these subjects and consequently non-detectable levels were reported in **Table 4.1**. Because the inhibitory effect is dependent on the Ara h 6 concentration (**Figure 4.2**) and because the optimal time point for determining the presence of Ara h 6 in serum is not yet known, we have evaluated the time course of appearance of Ara h 6 in serum for all 10 subjects from 30 min up to six hours, speculating that at some point in time the Ara h 6 would be high enough to overcome the inhibitory effect. **Figure 4.4** shows that only for the subjects whose serum did not inhibit the Ara h 6 ELISA, Ara h 6 could be detected at any time point.

Two key observations can be made from **Figure 4.4**. First, the detected levels of Ara h 6 seem to be fairly low. Considering the dose of Ara h 6 ingested with 100 g of peanuts (appr. 1.5 g), an average peak level of 0.34 ng/mL (± 0.20 ng/mL) after 60 min reflects only about 0.0001% of the intake at that moment in time. This low peak level in serum could be due to low uptake or to rapid clearance, or a combination of both. It is consistent with the view that the intestine functions as a barrier against the uptake of oligopeptides [27].

Second, the time point where the maximal concentration of Ara h 6 is found differs per subject. Because maximum levels are detected in serum after 120 min in most subjects this seems a suitable sampling time point for uptake detection, although a time course should be made for full quantification. Because all subjects have lower than peak levels after 4 h, a period up to 4–6 h after ingestion presents a good time-frame to capture the progress of allergen appearance in serum. This is in line with other reports on the appearance of food allergens in circulation upon ingestion [7, 8, 26].
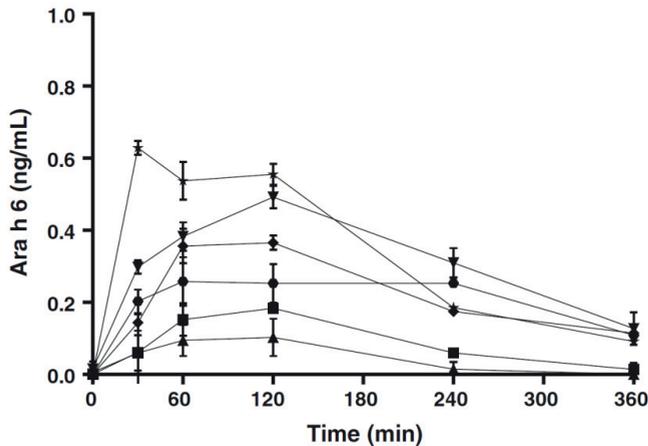
Figure 4.4. Naturally processed Ara h 6 detection in serum of study subjects after peanut ingestion

Ara h 6 ELISAs were performed to analyze the level of naturally processed Ara h 6 over time following 100 g of peanut intake. The values are shown as the average of two independent ELISAs. Markers are the same as in Figure 4.2. The AUC over time is on average 74.2 ± 42.1 ng·min/mL.

Implications for future research

Although literature describes some variation in peak levels of food allergens in the circulation after ingestion of allergen-containing foods, the levels are most often in the ng/mL range. As we demonstrate here, such low levels may be undetectable by immunochemical techniques in cases where the subjects have IgG/IgG4 against the relevant protein of interest. We utilise a sandwich ELISA, a common technique to detect and quantify allergens in complex matrices. A sandwich ELISA requires binding of at least two antibodies to two different epitopes of the allergen. Therefore, binding of an allergen to endogenous IgG/IgG4 may prevent reactivity in sandwich ELISA due to steric hindrance or occupation of amino acid sequences at or in close proximity to the antibody epitopes. ELISA formats that require only one antibody-analyte interaction (e.g. inhibition ELISA, direct ELISA, RAST inhibition [28]), may mathematically have a lower change for inhibition. The sensitivity of such formats is, however, not as good as that of sandwich ELISA, and possibly not sufficient to detect and quantify allergens in the ng/mL range. A very sensitive approach is the basophil histamine release assay. Dirks et al. [7] studied peanut allergen uptake using the basophil histamine release assay. While this was not designed as a quantitative assay, a dose-response effect was seen, and the time of peak presence of peanut protein was between 15 min and 2 h. Using the sensitivity of the assay (determined to be 5 pg/mL) the estimated concentration of peanut protein was in the higher pg/mL range, which is comparable to our observations. The basophil histamine release assay depends on allergen-antibody interaction, so the presence of IgG/IgG4 in the serum of the subjects could disturb

this assay as well. Because samples of 10 subjects were pooled in this study, it is not clear if individual inhibitory effects could have occurred.

Assays independent of analyte-antibody interaction may provide a way to circumvent such inhibitory effect. Mass spectrometry has been applied to detect cow's milk proteins [29] and peanut protein [13] in human breast milk, however the applied technique (i.e. peptide mass fingerprinting) is not quantitative. Truly quantitative mass spectrometry assays have been developed for peanut allergens, however these have not yet been applied for quantification of low concentrations of peanut allergens in serum or breast milk [30].

When comparing the concentration ranges reported for food allergens in serum with those of food allergens in breast milk, it seems that the concentrations in breast milk are higher. It can be hypothesized that peanut protein is transported via the circulation at low concentrations and is potentially circulated through the lymphatic system and excreted by the mammary gland, building up higher concentrations in breast milk. The role of IgG/IgG4 in this process is unknown. Bernard et al. [11] showed in a mouse model that Ara h 6, once in breast milk, could also interact with IgG. Whether this is also the case in humans needs to be further investigated. Also, the mechanism of the binding of IgG/IgG4 in relation to the transportation and clearance of food allergens in the circulation needs to be further investigated in order to better understand the relevance of intact food allergens (or large immunoreactive peptides of these allergens) circulating in serum after consumption.

Conclusions

We conclude that the presence of Ara h 6-specific IgG and IgG4 antibodies is a determining factor in the detection of Ara h 6 in serum. These antibodies, which are found in the blood circulation of healthy individuals and may be induced by consumption of peanut, interfere with the detection and quantification of Ara h 6 in serum. This may be a broader phenomenon in studies on the uptake of food allergens in circulation, and may explain why variable levels of food allergen in serum have been reported in literature.

Funding

Conflict of interest

The authors declare that they have no conflict of interest. For the avoidance of doubt, SJK reports to be consultant of DBV Technologies, a company involved in development of epicutaneous immunotherapy for food allergies, which is outside the scope of the research presented here.

References

1. Klemans, R.J., et al., The diagnostic accuracy of specific IgE to Ara h 6 in adults is as good as Ara h 2. Allergy, 2014. 69(8): p. 1112-1114.
2. Astwood, J.D., J.N. Leach, and R.L. Fuchs, Stability of food allergens to digestion *in vitro*. Nature biotechnology, 1996. 14(10): p. 1269-1273.
3. Pfeifer, S., et al., Cor a 14, the allergenic 2S albumin from hazelnut, is highly thermostable and resistant to gastrointestinal digestion. Molecular Nutrition & Food Research, 2015. 59(10): p. 2077-2086.
4. Sen, M., et al., Protein structure plays a critical role in peanut allergen stability and may determine immunodominant IgE-binding epitopes. The Journal of Immunology, 2002. 169(2): p. 882-887.
5. Koppelman, S.J., et al., Digestion of peanut allergens Ara h 1, Ara h 2, Ara h 3, and Ara h 6: A comparative *in vitro* study and partial characterization of digestion-resistant peptides. Molecular Nutrition & Food Research, 2010. 54(12): p. 1711-1721.
6. Apostolovic, D., et al., Conformational stability of digestion-resistant peptides of peanut conglutins reveals the molecular basis of their allergenicity. Scientific Reports, 2016. 6: p. 29249.
7. Dirks, C.G., et al., Does absorption across the buccal mucosa explain early onset of food-induced allergic systemic reactions? Journal of Allergy and Clinical Immunology, 2005. 115(6): p. 1321-1323.
8. Baumert, J., et al., Distribution of Intact Peanut Protein and Digestion-Resistant Ara h 2 Peptide in Human Serum and Saliva. Journal of Allergy and Clinical Immunology, 2009. 123(2): p. S268.
9. Husby, S., et al., Passage of dietary antigens into the blood of children with coeliac disease. Quantification and size distribution of absorbed antigens. Gut, 1987. 28(9): p. 1062-1072.
10. Matsuo, H., et al., Exercise and aspirin increase levels of circulating gliadin peptides in patients with wheat-dependent exercise-induced anaphylaxis. Clinical & Experimental Allergy, 2005. 35(4): p. 461-466.
11. Bernard, H., et al., Peanut allergens are rapidly transferred in human breast milk and can prevent sensitization in mice. Allergy, 2014. 69(7): p. 888-897.
12. Vadas, P., et al., Detection of peanut allergens in breast milk of lactating women. Journal of the American Medical Association, 2001. 285(13): p. 1746-1748.
13. Schocker, F., et al., Prospective investigation on the transfer of Ara h 2, the most potent peanut allergen, in human breast milk. Pediatric Allergy and Immunology, 2016. 27(4): p. 348-355.
14. Jacobs, J.F.M., et al., Anaphylaxis from Passive Transfer of Peanut Allergen in a Blood Product. New England Journal of Medicine, 2011. 364(20): p. 1981-1982.
15. Koppelman, S., et al., Purification and immunoglobulin E-binding properties of peanut allergen Ara h 6: evidence for cross-reactivity with Ara h 2. Clinical & Experimental Allergy, 2005. 35(4): p. 490-497.
16. Oerise, N.L., et al., Yield, proximate composition and mineral element content of three cultivars of raw and roasted peanuts. Journal of Food Science, 1974. 39(2): p. 264-266.
17. Koppelman, S.J., et al., Allergenicity attributes of different peanut market types. Food and Chemical Toxicology, 2016. 91: p. 82-90.
18. Bernard, H., et al., Identification of a new natural Ara h 6 isoform and of its proteolytic product as major allergens in peanut. Journal of Agricultural and Food Chemistry, 2007. 55(23): p. 9663-9669.
19. Husby, S., Normal immune responses to ingested foods. Journal of Pediatric Gastroenterology and Nutrition, 2000. 30(1): p. S13-S19.
20. Tay, S., et al., Patterns of immunoglobulin G responses to egg and peanut allergens are distinct: ovalbumin-specific immunoglobulin responses are ubiquitous, but peanut-specific immunoglobulin responses are up-regulated in peanut allergy. Clinical & Experimental Allergy, 2007. 37(10): p. 1512-1518.
21. Strait, R.T., S.C. Morris, and F.D. Finkelman, IgG-blocking antibodies inhibit IgE-mediated anaphylaxis *in vivo* through both antigen interception and FcγRIIb cross-linking. The Journal of Clinical Investigation, 2006. 116(3): p. 833-841.
22. Buchanan, A.D., et al., Egg oral immunotherapy in nonanaphylactic children with egg allergy. Journal of Allergy and Clinical Immunology, 2007. 119(1): p. 199-205.

23. Jones, S.M., et al., Clinical efficacy and immune regulation with peanut oral immunotherapy. Journal of Allergy and Clinical Immunology, 2009. 124(2): p. 292-300. e97.

24. Vickery, B.P., et al., Peanut oral immunotherapy modifies IgE and IgG 4 responses to major peanut allergens. Journal of Allergy and Clinical Immunology, 2013. 131(1): p. 128-134. e3.

25. Mohammed, I., et al., Multiple immune complexes and hypocomplementaemia in dermatitis herpetiformis and coeliac disease. Lancet, 1976. 308(7984): p. 487-490.

26. Husby, S., J.C. Jensenius, and S.E. SVEHAG, Passage of undegraded dietary antigen into the blood of healthy adults Further characterization of the kinetics of uptake and the size distribution of the antigen. Scandinavian Journal of Immunology, 1986. 24(4): p. 447-455.

27. Miner-Williams, W.M., B.R. Stevens, and P.J. Moughan, Are intact peptides absorbed from the healthy gut in the adult human? Nutrition Research Reviews, 2014. 27(02): p. 308-329.

28. Hamilton, R., Allergen-specific human IgE antibody based analysis of food, in Handbook of food allergen detection and control. 2014, Woodhead Publishing, London.

29. Coscia, A., et al., Cow's milk proteins in human milk. Journal of Biological Regulators and Homeostatic Agents, 2012. 26(3 Suppl): p. 39-42.

30. Johnson, P.E., et al., Quantitative Proteomic Profiling of Peanut Allergens in Food Ingredients Used for Oral Food Challenges. Analytical Chemistry, 2016.

Chapter 5

Endurance exercise increases intestinal uptake of the peanut allergen Ara h 6 after peanut consumption in humans

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Abstract

Controlled studies on the effect of exercise on intestinal uptake of protein are scarce and underlying mechanisms largely unclear. We studied the uptake of the major allergen Ara h 6 following peanut consumption in an exercise model and compared this with changes in markers of intestinal permeability and integrity. Ten overnight fasted healthy non-allergic men (n=4) and women (n=6) (23±4yrs) ingested 100 grams of peanuts together with a lactulose/rhamnose (L/R) solution, followed by rest or by 60 minutes cycling at 70% of their maximal workload. Significantly higher, though variable, levels of Ara h 6 in serum were found during exercise compared to rest (Peak P = 0.03; AUC P = 0.006), with individual fold changes ranging from no increase to an increase of over 150-fold in the uptake of Ara h 6. Similarly, uptake of lactulose (2-18 fold change, P = 0.0009) and L/R ratios (0.4-7.9 fold change, P = 0.04) were significantly increased which indicates an increase in intestinal permeability. Intestinal permeability and uptake of Ara h 6 were strongly correlated (r = 0.77, P <0.0001 for lactulose and Ara h 6). Endurance exercise after consumption may lead to increased paracellular intestinal uptake of food proteins.

Introduction

The food and Agriculture Organization of the United Nations predicted that the demand for animal-sourced foods will almost double between 2000 and 2030 due to the fast growth of the global population and changes in consumption patterns with an increase in urbanization in several parts of the world [1]. In order to close the gap between demand and supply, many research initiatives have been taken to study sustainable protein sources for food and feed [2-4]. In this quest, there is a strong focus on novel proteins derived from various sources such as plants, algae, food waste, and insects. Next to the technical criteria, questions that arise from the field of novel proteins are directed at their potential effects on human health, with respect to their digestibility and absorption [4], and their potency to activate the immune system [5]. The intestine is of major importance for the proper digestion and absorption of these novel proteins, but it also forms a protective barrier. Changes in intestinal barrier function (e.g. increased permeability) under different physiological conditions may result in increased absorption of large molecules including protein fragments [6-8], which could potentially induce an immune response. Mounting evidence indicates that increased intestinal permeability to antigens plays a key role in various inflammatory and autoimmune disorders, underlining the necessity to gain more insight into factors affecting intestinal barrier function [9].

The possibility that the intestine becomes permeable to certain macromolecules, presumably in a variable manner, has been acknowledged for some decades [8,10]. However, only very few studies in humans have investigated the uptake of intact dietary peptides and proteins in a controlled experimental setting [11].

An increased intestinal permeability to immune-reactive proteins or their fragments as a result of intensive exercise has also been postulated as a possible mechanism underlying food-dependent exercise-induced anaphylaxis [12,13].

The aim of the present study was to substantiate the increase in intestinal permeability for dietary proteins upon exercise by determining the effects of a controlled exercise protocol on the uptake kinetics and levels of the peanut allergen Ara h 6 following peanut consumption and to relate this to markers of intestinal permeability and integrity. Ara h 6 was chosen, because, together with Ara h 2, it is the major peanut allergen. Furthermore, Ara h 6 was shown to be resistant to digestion, which increases the possibility of detecting this specific protein in the circulation [14].

Subjects and methods

Ethical approval

This study was approved by the medical ethical committee of Wageningen University (METC-WU 16/11; date of approval: 10 May 2016; Netherlands trial register: NTR5854, PEANUTS Study), and conducted in accordance with the Declaration of Helsinki. All subjects gave their

written informed consent after the set-up, objectives and possible consequences of the study had been fully explained.

Subjects

Ten healthy non-atopic subjects, based on exercise-induced effects on intestinal permeability, were recruited via social media and the email database of the university's Division of Human Nutrition (Wageningen University and Research, NL). Exclusion criteria were exercise for > three hours per week, smoking, use of drugs, use of nonsteroidal anti-inflammatory drugs (NSAIDs) on a chronic basis, or the use of any medication for gastric or intestinal complaints. Subjects did not perform intense physical activity three days prior to the test days. Subjects also did not eat peanut-containing foods one day nor used alcohol two days prior to the test days. Characteristics of the 10 subjects included in this study are shown in **Table 5.1**.

Table 5.1. Subject characteristics

ID	M/F	Age (years)	BMI (kg/m^2)	Wmax (watt)	Consumption of products with high peanut content[1]	Consumption of products with low peanut content[2]
1	F	22	24.9	240	1x per week	<1x per month
2	F	21	22.7	200	<1x per month	1-3x per month
3	F	22	25.6	320	1-3x per month	1-3x per month
4	F	22	25.2	280	(Almost) daily	1-3x per month
5	M	20	24.8	320	1x per week	1-3x per month
6	M	21	24.7	360	1x per week	1x per week
7	M	26	22.7	300	1-3x per month	<1x per month
8	M	32	23.5	300	1x per week	<1x per month
9	F	22	22.8	260	(Almost) daily	<1x per month
10	F	23	23.6	200	(Almost) daily	1-3x per month

[1] Examples: peanuts/ peanut butter/ peanut sauce [2] Examples: candy bars/ cookies/ muesli (bars) Wmax = Maximal cycling workload M/F = Male/Female BMI = Body Mass Index

Pre-study assessment

Pre-screening was performed prior to subject inclusion to prevent inclusion of subjects in whose serum no Ara h 6 would be reliably detected after peanut intake due to inhibiting effects of the serum matrix; e.g. because of the presence of Ara h 6-specific IgG and particularly IgG4 in the serum, which was found to be common in our study population [14]. This was tested with an in-house developed enzyme-linked immunosorbent assay (ELISA) [14]. Fasted blood samples were tested by spiking 3.1 ng/mL Ara h 6 in 1:1 diluted serum samples. Subjects with a spike recovery <50% were excluded from participation.

A maximal aerobic capacity test (Wmax) was performed using an electronically braked cycle ergometer (Lode Excalibur, Groningen, NL). After a short warm-up, the subjects started cycling at 100W. Every minute the power increased with 20W until they were no longer able to maintain the workload (Wmax; Pedal frequency falling from 90-100 rotations per minute to less than 70).

Study design

Each subject was tested once in rest and once during and following exercise. Test sessions at the university always started after an overnight fast. Pre-ingestion blood was sampled, after which 100 grams of mildly-roasted, unsalted peanuts, obtained from a local supermarket (Albert Heijn; private label product) were consumed within 10 minutes. This was directly followed by the intake of a solution of two inert, non-digestible, test sugars, containing one gram of L-rhamnose (Carbosynth, Compton, Berkshire, UK) and 0.5 grams of lactulose (Carbosynth) dissolved in 100 mL tap water. Participants were seated comfortably (rest). Blood was sampled in ethylenediaminetetraacetic acid (EDTA) plasma tubes (Vacutainer, Becton Dickinson, Breda, NL) as well as serum separator tubes (Vacutainer) at an interval of 30, 60, 120, 240, and 360 minutes after their peanut consumption. EDTA blood tubes were directly centrifuged at 2000× g for 10 minutes at 4°C, after which plasma was aliquoted and stored at -80°C. Serum separator tubes were left to clot for at least 20 minutes at room temperature, after which the tubes were centrifuged at 2000× g for 10 minutes at room temperature to obtain serum, which was directly aliquoted and also stored at -80°C until further analysis. Analysis of Ara h 6 was performed in blood samples collected at t= 0, 30, 60, 90, 120, and 240 min. Analysis of fatty acid binding protein-2 (FABP2) was performed in blood samples collected at t= 0, 30, and 60 min. Analysis of lactulose and rhamnose was performed in blood samples collected at t= 0 and 60 min. One week later, the same procedure was followed, yet this time the intake of the peanuts and the dual sugar solution was followed by endurance exercise. The endurance exercise involved individuals cycling for 60 minutes at 70% of their Wmax, followed by rest for the remaining three hours. Again, blood was sampled in EDTA plasma tubes as well as serum separator tubes 30, 60, 90 120, and 240 minutes after finishing their peanut consumption. See **Figure 5.1** for a schematic overview of the intervention and blood sampling.
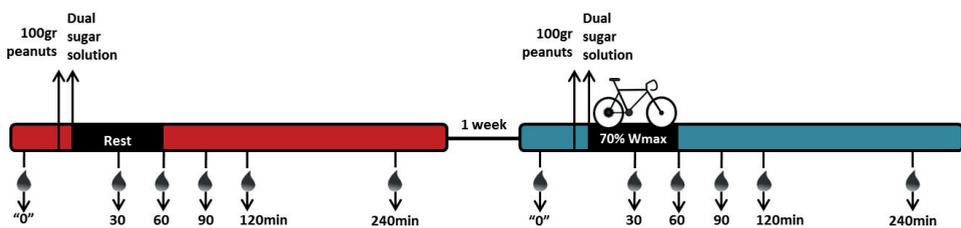

Figure 5.1. Schematic overview of study set up
This scheme shows the blood sampling during Rest and Exercise. Black box depicts the intervention: 60 minutes of rest or 60 min of cycling at 70% of the individual's maximal cycling workload (Wmax). The analyses performed with blood sampled at the different time points are listed. FABP2= fatty acid binding protein

Peanut allergen Ara h 6 detection in serum

Serum levels of Ara h 6 were determined with an Ara h 6 ELISA from Indoor Biotechnologies (EL-AH6, Cardiff, UK). The instructions of the manufacturer were followed, except for the reference material, which was replaced by naturally processed Ara h 6, as it occurs next to

its native intact form in peanuts [14]). This form of Ara h 6 supposedly better represents Ara h 6 after gastrointestinal digestion [15]. This naturally processed Ara h 6 has been thoroughly tested previously and has shown good reactivity in the assay [14]. The lower level of quantification of Ara h 6 was 0.024 ng/mL. Samples were tested in duplicate and ELISA analyses were performed twice.

Parameters of intestinal permeability and integrity

To assess intestinal permeability, levels of lactulose and L-rhamnose were analyzed in plasma sampled at t=0 and 60 minutes after intake of the peanuts and the dual sugar solution.

To this end, 125 µL EDTA plasma was transferred to Eppendorf tubes equipped with a 3 kDa cut-off filter (Amicon Ultra 0.5 mL 3K, Millipore, Etten-Leur, The Netherlands) to remove the plasma proteins. The filter tubes were centrifuged for 30 minutes at 11000g at 4°C, and clear plasma filtrate was inserted in the pre-cooled sample processor (233 XL, Gilson, Middleton, WI, USA). Sugars were subsequently analysed by LC-MS as described [16]. Plasma lactulose/rhamnose (L/R) ratios were calculated from the 60 minutes plasma concentrations that were corrected for findings at baseline.

FABP2 was measured as an acute marker of small intestinal epithelial integrity in EDTA plasma (1:1) with an in-house developed ELISA, as described [17].

Statistics

All data is shown as mean ± standard deviation. The Wilcoxon matched-pairs signed rank test was applied to assess changes in AUC, maximal peak height, and time-to-peak analyses of serum levels of Ara h 6 between rest and exercise. Paired T-test was used to assess changes in lactulose, rhamnose, and L/R ratio between rest and exercise. Log transformation was applied in case of a deviation from a Gaussian distribution. For rhamnose and L/R ratio analyses, one person was excluded, because no rhamnose could be detected in one of the samples. Two-way ANOVA with Bonferroni's multiple comparisons test was used to assess differences in FABP2 levels over time between rest and exercise. Pearson's correlation coefficients were used to correlate the Ara h 6 parameters, intestinal permeability parameters, and FABP2 levels. All statistics were performed with GraphPad Prism (version 6.07, GraphPad Software Inc., San Diego, CA, USA). Statistical significance was defined as a two-tailed $P < 0.05$.

Results

Exercise increases the levels of Ara h 6 in serum

In rest, consumption of 100 grams of peanuts resulted in detectable serum Ara h 6 levels in each of the subjects (**Figure 5.2A**). These levels increased when the peanut consumption was followed by exercise, with large intra-individual differences (**Figure 5.2B**). One out of the ten

subjects did not show an increase in the peak level of Ara h 6 in exercise compared to rest (**Figure 5.2C**), while the other subjects did display an increase. Fold changes ranged from 0.7 to 140.6 and the median peak Ara h 6 increased significantly from 0.22 in rest to 0.55 ng/mL during exercise (P = 0.01). The AUC calculated from time point t=0 to t=240 also changed significantly (P = 0.006) as the median AUC increased from 34.63 ng·min/mL in rest to 77.10 ng·min/mL in exercise (**Figure 5.2D**). Here, individual fold changes ranged from 0.8 to 155.1 fold. Compared to rest, the peak concentration of Ara h 6 in serum after peanut intake was reached earlier in time (P = 0.004) during exercise (**Figure 5.2E**).
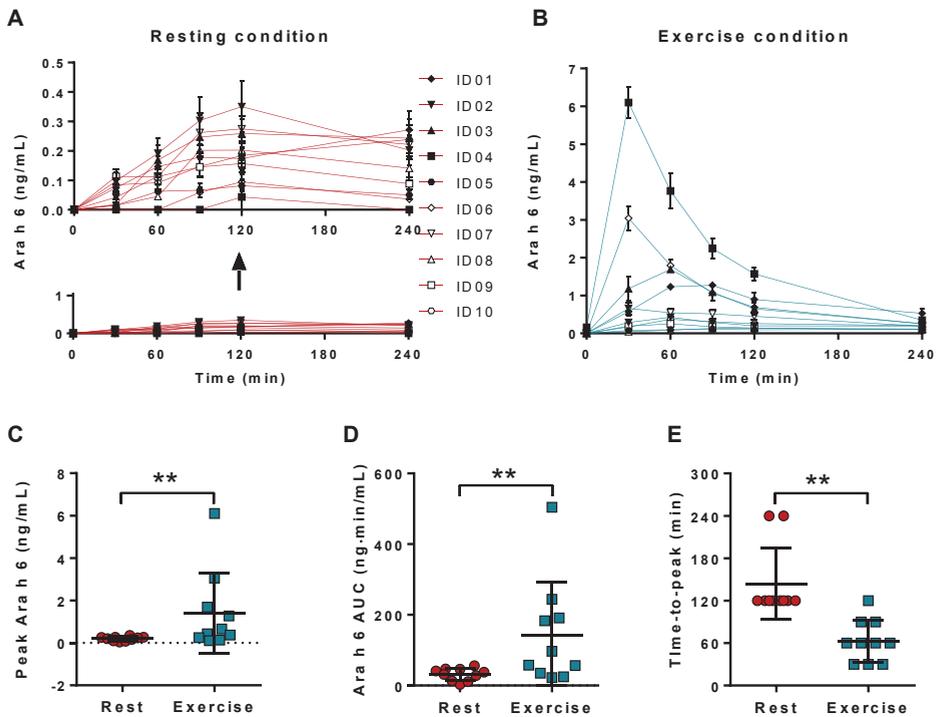
Figure 5.2. Ara h 6 levels in serum after peanut intake following Rest or Exercise
Serum levels of Ara h 6 (Mean ± standard deviation) was measured 30, 60, 90, 120, and 240 min after the consumption of 100 grams of peanuts. This intake was followed by rest (A, red lines) or by exercise (60 minutes of cycling at 70% Wmax) (B, blue lines). For both conditions, individual peak levels (C), AUC (D), and time-to-peak (E) were determined for rest (red circle) and exercise (blue square). * represents a P-value < 0.05; ** represents a P-value <0.01

Exercise-induced effects on intestinal permeability and integrity markers

Exercise-induced changes in integrity of the small intestine can be experimentally assessed by its permeability to small compounds, such as the inert sugar lactulose [18-21]; and by

circulating markers of intestinal integrity, such as FABP2 [22]. Plasma levels of lactulose increased significantly (P = 0.0009) when consumption of peanuts is followed by exercise compared to rest (**Figure 5.3A**). There was no difference in the absorption of L-rhamnose (Figure 3B), although one could see a tendency towards increased absorption (P = 0.08). Nevertheless, the calculated L/R ratios showed a significant increase (P = 0.04) as individual ratio fold changes ranged from 0.4 to 7.9-fold. These results suggest an increase in paracellular intestinal permeability when consumption of peanuts is followed by exercise.

The effect of peanut consumption and endurance exercise on intestinal integrity was determined with circulating levels of FABP2. **Figure 5.4** shows that plasma FABP2 levels were increased significantly after 30 minutes of exercise compared to the levels when the peanuts were consumed followed by rest. This increase became more pronounced after 60 minutes of exercise when compared to their values during rest.
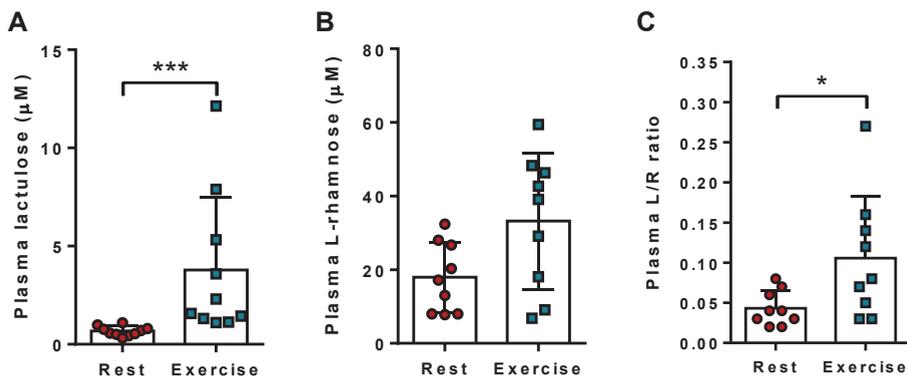
Figure 5.3. Lactulose and L-rhamnose sugar test for the analysis of intestinal permeability during Rest or Exercise
Plasma levels of lactulose (A) and L-rhamnose (B) (Mean ± standard deviation) were measured after intake of the dual sugar solution followed by rest or exercise. From these, the Lactulose-over-l-rhamnose (L/R) ratio was calculated (C). * represents a P-value < 0.05; *** represents a P-value <0.001
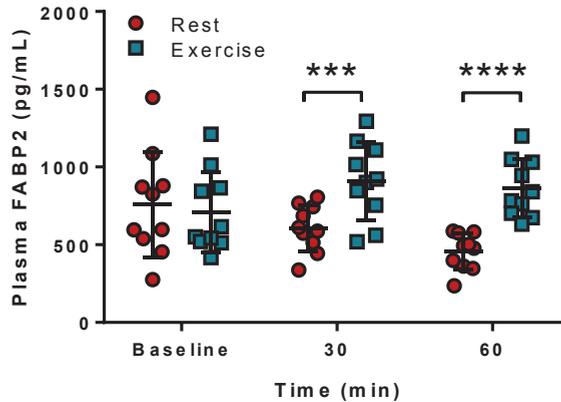
Figure 5.4. Circulating fatty acid binding protein-2 (FABP2) levels upon exercise

Fatty acid binding protein-2 levels (Mean ± standard deviation) were measured in plasma as a marker of intestinal integrity after 30 minutes or 60 minutes during rest (red circles) or exercise (blue squares). *** represents a P-value <0.001; **** represents a P-value <0.0001

Correlations of Ara h 6 levels and intestinal permeability and integrity markers

Pearson correlation coefficients were calculated to determine the correlation between the uptake of Ara h 6 and intestinal permeability as reflected by the uptake of lactulose. There was a strong correlation between the lactulose levels both with the peak levels of Ara h 6 (r = 0.77; P <0.0001; **Figure 5.5A**) and with the Ara h 6 AUC values (r = 0.70; P = 0.0005; **Figure 5.5B**). The L/R ratio also correlated well with the peak levels of Ara h 6 (r = 0.66; P = 0.0007; **Figure 5.5D**) and the Ara h 6 AUC values (r = 0.60; P = 0.002; **Figure 5.5E**). The correlations between FABP2 levels after 60 minutes and lactulose levels (r = 0.58, P = 0.008; **Figure 5.5C**), L/R ratio (r = 0.54, p = 0.04; **Figure 5.5F**), Ara h 6 peak levels (r = 0.55, p = 0.01; **Figure 5.5G**), and Ara h 6 AUC (r = 0.60, P = 0.005; **Figure 5.5H**) were slightly lower, but still significant.
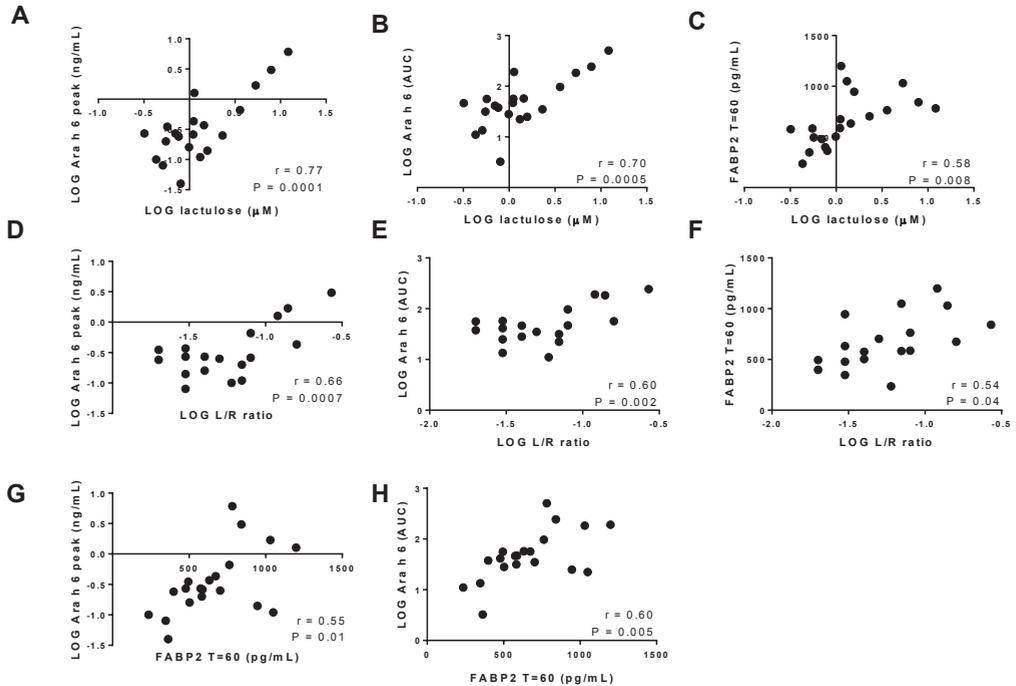
Figure 5.5. Correlation analysis of intestinal integrity markers and levels of Ara h 6

Correlations are shown with the calculated Pearson's correlation coefficients (r) and statistical significance. Correlations are made between the lactulose levels and the peak levels of Ara h 6 A) and the Ara h 6 AUC values (B), between the Lactulose-over-l-rhamnose (L/R) ratio and the peak levels of Ara h 6 (D) and the Ara h 6 area under the curve (AUC) values (E), and between fatty acid binding protein 2 (FABP2) levels after 60 minutes and lactulose levels (C), L/R ratio (F), Ara h 6 peak levels (G), and Ara h 6 AUC (H). LOG = logarithm.

Discussion

The aim of the present study was to determine the effects of endurance exercise on intestinal permeability to the peanut-derived allergen Ara h 6 and to compare this with commonly used small molecule markers for intestinal permeability. Although exercise-induced permeability towards potential allergens has been hypothesized to play a role in food-dependent exercise-induced anaphylaxis, the experimental evidence in humans is limited so far [23,24].

The current study shows a clear increase in the level of Ara h 6 as measured by ELISA in serum when peanut consumption was followed by exercise compared to the resting condition. Cycling at 70% Wmax also resulted in increased lactulose levels and consequently increased L/R ratios compared to rest. This outcome supports the hypothesis that exercise leads to increased paracellular permeability of the small intestine towards small molecules

[25]. The present study furthermore indicates a high correlation between the levels of Ara h 6 and those of lactulose. It is therefore enticing to suggest a direct role for increased intestinal paracellular permeability in absorption of this allergen. There is a rather high variation in effect between the individuals and these differences appear to be similar for the effects on the levels of Ara h 6 as well as the L/R ratio. The causes for such inter-individual differences are not known.

Although assumptions are often made, there is limited evidence besides our study for the passage of larger food-derived proteins and peptides across the intestinal barrier into the circulation [6,26-30], in particular in combination with exercise-induced permeability. Matsuo et al. [12] showed increased levels of circulating gliadin peptides upon exercise and intake of NSAIDs, but did not assess intestinal permeability. In recent studies, we used a strenuous exercise protocol involving both a glycogen depletion interval phase and an endurance exercise phase, which when compared to resting conditions, resulted in an increase in intestinal permeability to lactulose [31,32]. In the same model we detected an exercise-induced increase of the urinary recovery of the small casein-derived peptide betacasomorphin-7 [32]. However, the question for the uptake of larger dietary protein in the blood in combination with exercise remained. It has been demonstrated that the peanut protein Ara h 2, which together with Ara h 6, are the most potent peanut allergens [32,34], can be detected in breast milk after peanut ingestion [35], but detection in blood has not yet been proven and the effect of exercise not studied. Although peanut consumption and Ara h 6 detection are included in the resting condition, performing an exercise-only intervention could additionally provide information on a possible interaction between peanut consumption and exercise on intestinal permeability.

Because little is known on the absorption and clearance mechanisms of Ara h 6, or its derived peptides, in principle the increase in serum Ara h 6 peak levels could result from increased passage across the intestinal epithelial barrier, from lower clearance rate, or a combination of the two. However, given the effects seen on markers for intestinal permeability, and the strong correlation between those parameters, an increased passage seems conceivable. Theoretically, changes in intestinal digestion due to exercise could lead to changes in the detection of Ara h 6. It seems likely, however, that the allergen is crossing the barrier relatively intact. Digestion of Ara h 6 by pepsin and trypsin has been investigated in the past, showing that these only partially hydrolyse Ara h 6, resulting in two large peptides of around 4 to 5 kDa and 10 kDa in size, which are bound to each other by disulphide bridges [15,36,37]. We cannot claim with certainty whether it is the intact Ara h 6 protein or these two bridged peptides that are appearing in the serum. These peptides are comparably reactive with intact Ara h 6 in the ELISA method that has been used [14]. The uptake of Ara h 6 and its immunoreactive fragments that are detected are physiologically relevant as these fragments are thought to contribute to the allergenicity of peanuts. Ara h 1 and Ara h 3 are much more sensitive to digestion compared to Ara h 6 and Ara h 2, making them less able to trigger an immune response [36,38,39]. This study reports for the first time the detection of Ara h 6 in the blood. Although physiological levels of Ara h 6 are not exactly

known, the levels found in the current study is comparable to the levels of Ara h 2 in the circulation after peanut intake found by others [30,38].

In most individuals, the highest Ara h 6 level in serum was found 120 minutes after peanut intake, which is similar in time as found in our prior study [14]. This peak, however, clearly shifted to earlier time points when the intake of peanuts was followed by intense exercise. This is possibly due to an increased gastric emptying rate of a solid meal during exercise [40]. Alternatively, this may be related to a forward shift in steady state [41] or changes in the route of epithelial transport.

In the current study, a median peak level of 0.55 ng/mL during exercise reflects about 0.0002% of the ingested dose of Ara h 6. This level lies within the suggested scope of antigen sampling, which takes place via transcytosis and sampling by dendritic cells. This process is estimated to occur at rates which are several orders of magnitude smaller than 0.1% of the administered dose [10,42]. These values, however, are mostly based on animal models or *in vitro* testing and only limited data in humans is available. The occurrence of food-dependent exercise-dependent anaphylaxis suggests that increased absorption during exercise is clinically relevant, as the threshold for allergic symptoms could be reached more easily at a similar intake.

In conclusion, we show that endurance exercise after peanut consumption results in a clear increase in the serum levels of Ara h 6 after peanut consumption, which correlates strongly with increased intestinal permeability. This suggests that exercise has a profound effect on the amount of food-derived allergen crossing the epithelial barrier, also in healthy non-sensitized individuals. This effect is possibly mediated by an increase in intestinal permeability. The current model could be used to screen for the potential health effects of novel protein sources under different physiological conditions.

Conflict of interest

The authors declare that they have no conflict of interest.

Authors' contributions to manuscript

LJ, KN, SG, SK, JK, RW, and HW conceived and designed the experiments; LJ performed the experiments; LJ, KL analyzed the data; SK contributed reagents; LJ, KN, SG, JK, RW and HW wrote the paper. All authors read, critically revised and approved the final manuscript.

Chapter 5

References

1. Robinson, T. and F. Pozzi, Mapping supply and demand for animal-source foods to 2030. Animal Production and Health Working Paper, 2011. 2.
2. Aiking, H., Future protein supply. Trends in Food Science & Technology, 2011. 22(2–3): p. 112-120.
3. Smil, V., Worldwide transformation of diets, burdens of meat production and opportunities for novel food proteins. Enzyme and Microbial Technology, 2002. 30(3): p. 305-311.
4. Boland, M.J., et al., The future supply of animal-derived protein for human consumption. Trends in food science & technology, 2013. 29(1): p. 62-73.
5. Ladics, G., et al., Review of animal models designed to predict the potential allergenicity of novel proteins in genetically modified crops. Regulatory Toxicology and Pharmacology, 2010. 56(2): p. 212-224.
6. Lambert, G., Intestinal barrier dysfunction, endotoxemia, and gastrointestinal symptoms: the 'canary in the coal mine'during exercise-heat stress?, in Thermoregulation and Human Performance. 2008, Karger Publishers. p. 61-73.
7. Gardner, M.L.G., Passage of intact peptides across the intestine. Advances in the Biosciences, 1987. 65: p. 99-106.
8. Gardner, M.L.G., Gastrointestinal absorption of intact proteins. Annual review of nutrition, 1988. 8(1): p. 329-350.
9. Vojdani, A., For the assessment of intestinal permeability, size matters. Alternative Therapies In Health And Medicine, 2013. 19(1): p. 12-24.
10. Weiner, M., Intestinal transport of some macromolecules in food. Food and Chemical Toxicology, 1988. 26(10): p. 867-880.
11. Miner-Williams, W.M., B.R. Stevens, and P.J. Moughan, Are intact peptides absorbed from the healthy gut in the adult human? Nutrition Research Reviews, 2014. 27(02): p. 308-329.
12. Matsuo, H., et al., Exercise and aspirin increase levels of circulating gliadin peptides in patients with wheat-dependent exercise-induced anaphylaxis. Clinical & Experimental Allergy, 2005. 35(4): p. 461-466.
13. Barg, W., W. Medrala, and A. Wolanczyk-Medrala, Exercise-induced anaphylaxis: an update on diagnosis and treatment. Current Allergy and Asthma Reports, 2011. 11(1): p. 45-51.
14. JanssenDuijghuijsen, L.M., et al., Detection of peanut allergen in human blood after consumption of peanuts is skewed by endogenous immunoglobulins. Journal of Immunological Methods, 2017. 440: 52-57.
15. Apostolovic, D., et al., Conformational stability of digestion-resistant peptides of peanut conglutins reveals the molecular basis of their allergenicity. Scientific Reports, 2016. 6: p. 29249.
16. van Wijck, K., et al., Novel analytical approach to a multi-sugar whole gut permeability assay. Journal of Chromatography B: Biomedical Applications, 2011. 879(26): p. 2794-2801.
17. Van Wijck, K., et al., Aggravation of exercise-induced intestinal injury by Ibuprofen in athletes. Medicine and Science in Sports and Exercise, 2012. 44(12): p. 2257-2262.
18. Marchbank, T., et al., The nutriceutical bovine colostrum truncates the increase in gut permeability caused by heavy exercise in athletes. American Journal of Physiology - Gastrointestinal and Liver Physiology, 2011. 300(3): p. G477-G484.
19. Øktedalen, O., et al., Changes in the gastrointestinal mucosa after long-distance running. Scandinavian Journal of Gastroenterology, 1992. 27(4): p. 270-274.
20. Pals, K.L., et al., Effect of running intensity on intestinal permeability. Journal of Applied Physiology, 1997. 82(2): p. 571-576.
21. Van Wijck, K., et al., Exercise-induced splanchnic hypoperfusion results in gut dysfunction in healthy men. PLoS ONE, 2011. 6(7): p. e22366.
22. Pelsers, M.M.A.L., W.T. Hermens, and J.F.C. Glatz, Fatty acid-binding proteins as plasma markers of tissue injury. Clinica Chimica Acta, 2005. 352(1–2): p. 15-35.

23. Price, D., L. Ackland, and C. Suphioglu, Nuts'n'guts: transport of food allergens across the intestinal epithelium. Asia Pacific Allergy, 2013. 3(4): p. 257.

24. Price, D.B., et al., Peanut Allergens Alter Intestinal Barrier Permeability and Tight Junction Localisation in Caco-2 Cell Cultures. Cellular Physiology and Biochemistry, 2014. 33(6): p. 1758-1777.

25. Bjarnason, I., A. Macpherson, and D. Hollander, Intestinal permeability: an overview. Gastroenterology, 1995. 108(5): p. 1566-1581.

26. Castell, J.V., et al., Intestinal absorption of undegraded proteins in men: presence of bromelain in plasma after oral intake. American Journal of Physiology - Gastrointestinal and Liver Physiology, 1997. 273(1): p. G139-G146.

27. Chabance, B., et al., Casein peptide release and passage to the blood in humans during digestion of milk or yogurt. Biochimie, 1998. 80(2): p. 155-165.

28. Husby, S., J.C. Jensenius, and S.E. Svehag, Passage of Undegraded Dietary Antigen into the Blood of Healthy Adults. Scandinavian Journal of Immunology, 1985. 22(1): p. 83-92.

29. Sokolov, O., et al., Autistic children display elevated urine levels of bovine casomorphin-7 immunoreactivity. Peptides, 2014. 56: p. 68-71.

30. Baumert, J.L., et al., Distribution of Intact Peanut Protein and Digestion-Resistant Ara h 2 Peptide in Human Serum and Saliva. Journal of Allergy and Clinical Immunology, 2009. 123(2, Supplement): p. S268.

31. JanssenDuijghuijsen, L.M., et al., Adaptation of exercise-induced stress in well-trained healthy young men. Experimental Physiology, 2017. 102: 86-99.

32. JanssenDuijghuijsen, L.M., et al., The effect of endurance exercise on intestinal integrity in well-trained healthy men. Physiological Reports, 2016. 4(20): e12994.

33. Flinterman, A., et al., Children with peanut allergy recognize predominantly Ara h 2 and Ara h 6, which remains stable over time. Clinical & Experimental Allergy, 2007. 37(8): p. 1221-1228.

34. Klemans, R.J., et al., The diagnostic accuracy of specific IgE to Ara h 6 in adults is as good as Ara h 2. Allergy, 2014. 69(8): p. 1112-1114.

35. Vadas, P., et al., Detection of peanut allergens in breast milk of lactating women. Journal of the American Medical Association, 2001. 285(13): p. 1746-1748.

36. Koppelman, S.J., et al., Digestion of peanut allergens Ara h 1, Ara h 2, Ara h 3, and Ara h 6: A comparative *in vitro* study and partial characterization of digestion-resistant peptides. Molecular Nutrition & Food Research, 2010. 54(12): p. 1711-1721.

37. Sen, M., et al., Protein structure plays a critical role in peanut allergen stability and may determine immunodominant IgE-binding epitopes. The Journal of Immunology, 2002. 169(2): p. 882-887.

38. Baumert, J.L., et al., Development of an ELISA for Digestion-Resistant Ara h 2 Peptide: Monitoring the Peptide in Body Fluids of Healthy Volunteers Who Consumed Peanut. Journal of Allergy and Clinical Immunology, 2006. 117(2, Supplement): p. S35.

39. Zhuang, Y. and S.C. Dreskin, Redefining the major peanut allergens. Immunologic Research, 2013. 55(1-3): p. 125-134.

40. Moore, J.G., F.L. Datz, and P.E. Christian, Exercise increases solid meal gastric emptying rates in men. Digestive Diseases and Sciences, 1990. 35(4): p. 428-432.

41. Greenberg, H.E., et al., Time of peak drug concentration after a single dose and a dose at steady state. The Journal of Clinical Pharmacology, 1997. 37(6): p. 480-485.

42. Paganelli, R. and R. Levinsky, Solid phase radioimmunoassay for detection of circulating food protein antigens in human serum. Journal of Immunological Methods, 1980. 37(3): p. 333-341.

Chapter 6

Intestinal epithelial integrity is dependent on mitochondrial ATP production: An *in vitro* model with an oxidative metabolic phenotype

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Abstract

Aim: *In vivo* studies suggest that maintenance of intestinal barrier function is dependent on mitochondrial ATP production. In this paper, we aim to provide mechanistic support for this observation using an *in vitro* model mimicking the oxidative *in vivo* situation.

Methods: Human Caco-2 cells were cultured for 10 days in cell culture flasks or for 14 days on transwell inserts in either glucose or galactose containing medium. Mitochondria were visualized and cellular respiration and levels of oxidative phosphorylation (OXPHOS) proteins were determined. Cells were treated with CCCP, rotenone, or piericidin A (PA) for up to 30 hours to induce cellular ATP depletion. Monolayer permeability was assessed with transepithelial electrical resistance (TEER) and fluorescein salt (FS) flux. Changes in gene expression of genes related to tight junctions were analysed.

Results: Caco-2 cells cultured in galactose, but not glucose, containing medium showed increased mitochondrial connectivity, oxygen consumption rates and levels of OXPHOS proteins. Inhibiting mitochondrial activity with CCCP, rotenone and piericidin A (PA) resulted in a dose-dependent increased monolayer permeability (decrease in TEER, increase in fluorescein flux). In-depth studies with PA showed an almost six fold decrease in cellular ATP and altered gene expression profiles of tight junction proteins 1 and 2, occludin, and claudins 1, 2 and 7.

Conclusions: Well-functioning mitochondria are essential for maintaining Caco-2 cellular energy status and monolayer integrity when grown on galactose medium.

Introduction

The gut is the major organ at the interface of the internal and external environment and plays an important role in nutrient uptake and metabolism, metabolic homeostasis and health. The splanchnic region is highly vascularized, which is pivotal to support the high metabolic demands of proper gut functioning [1]. The enterocytes lining the gut barrier are very sensitive to ischemic conditions [2]. Intestinal ischemia results in a state of inflammation and increased intestinal permeability [3, 4]. This suggests that oxidative energy metabolism plays an essential role in maintaining a gut barrier with high integrity. Several *in vivo* studies indirectly support this hypothesis, such as exercise studies that show an exercise-induced splanchnic hypoperfusion in combination with exercise-induced intestinal permeability [5]. Fructooligosaccharide supplementation in rats furthermore resulted in an increase in intestinal permeability [6]. Although in this study intestinal cellular energy status was not assessed, mitochondrial genes were highly regulated in a way similar to that induced by mitochondrial uncouplers. Finally, non-steroid anti inflammatory drugs are thought to induce intestinal permeability at least in part by uncoupling of mitochondria [7-9]. These findings together suggest an essential role for mitochondrial ATP production in maintaining a gut barrier with high integrity.

The Caco-2 cell line can be used to study the direct relation between mitochondrial ATP production and intestinal permeability. This human intestinal epithelial cell model, derived from a colon adenocarcinoma, demonstrates many *in vivo* intestinal functions and is the most widely used model to study the epithelial barrier function [10]. Good correlations have been reported between *in vitro* Caco-2 cell permeability and *in vivo* absorption in humans [11]. These cells, however, are routinely cultured in high glucose conditions thereby relying mainly on glycolysis for their ATP production. For several cell types it has been shown that an *in vitro* energy substrate switch from glucose to galactose results in a cellular phenotype relying more on mitochondrial ATP production [12-15]. Such an *in vitro* substrate switch is an improved representation of the *in vivo* situation and would enable one to study the role of mitochondrial ATP production in maintaining a healthy gut barrier. Therefore, our aim was to develop an *in vitro* intestinal permeability model mimicking the *in vivo* situation and, more crucially, to determine the role of mitochondrial ATP production in intestinal permeability.

Material and methods

Caco-2 cell culture

The Caco-2 human colon cancer cell line was obtained from the American Type Culture Collection (ATCC HTB-37TM, Manassas, VA, USA). These cells were routinely grown in 75 cm2 culture flasks (Corning, Amsterdam, NL) in high-glucose (25 mM) Dulbecco's Modified Eagle's medium (DMEM-glucose, Gibco, Breda, NL) supplemented with 100 U/L penicillin and 100 µg/L streptomycin (Gibco), 1 mM Na-pyruvate (Gibco), 2 mM glutamax (Gibco), 25

mM HEPES buffer (Gibco) and 10% heat-inactivated (56°C, 45 min) foetal bovine serum (FBS, HyClone, Thermo Scientific, Breda, NL). Caco-2 cells were cultured at 37°C in a humidified atmosphere with 5% CO2 up to 80% confluency and then either subcultured or used for the experiments. For all experiments, Caco-2 cells with a passage number between 30 and 36 were proliferated or differentiated using either DMEM-glucose or DMEM-galactose (glucose-free DMEM (GIBCO) supplemented with 25 mM galactose as well as the other supplements as in DMEM-glucose.

Mitochondrial respiration of Caco-2 cells

Proliferating Caco-2 cells were cultured in DMEM-glucose or DMEM-galactose for 10 days after which oxygen consumption rate (OCR) was measured using 1.0×10^6 cells. OCR was measured at 37°C using polygraphic oxygen sensors in a two-chamber Oxygraph (Oroboros Instruments, Innsbruck, AT). Basal OCR was determined first followed by 1) leak OCR: 1 µM oligomycin (Sigma Aldrich), blocking complex V of the OXPHOS; 2) maximal OCR: titration with 1 µM carbonyl cyanide m-chlorophenylhydrazone (CCCP, Abcam Cambridge, UK), uncoupling OXPHOS; 3) non-mitochondrial OCR: 1 µM antimycin A (Sigma Aldrich) and 1 µM rotenone (ROT, Sigma Aldrich), blocking complex III and I, respectively. This experiment was performed six times.

Caco-2 cells were also cultured in the XF96 cell culture microplate using DMEM-glucose or DMEM-galactose for 14 days to induce (semi-)differentiation. OCR was measured with the XF96 Extracellular Flux Analyzer (Seahorse Bioscience, Billerica, MA, USA). Basal OCR was determined first, followed by leak OCR: 15 µM oligomycin; maximal OCR: 1 µM carbonyl cyanide-p-trifluoromethoxyphenyl- hydrazone (FCCP, Sigma Aldrich); non-mitochondrial OCR: 25 µM antimycin A and 12.5 µM ROT. The FCCP concentration used was obtained from a titration experiment. The basal, leak, and maximal OCR values were corrected for non-mitochondrial OCR.

Mitochondrial visualization in proliferating Caco-2 cells

To visualize the mitochondria, Caco-2 cells were seeded in 35 mm plastic dishes (Eppendorf, Nijmegen, NL) at $0.3\text{-}1.0 \times 10^4$ cells per cm2 in DMEM-glucose or DMEM-galactose and cultured for 10 days when 70% confluency was reached. Then, culture medium was replaced by phenol red-free DMEM (supplemented as DMEM-glucose or DMEM-galactose) with 200nM MitoTrackerTM Green FM (Life Technologies, Breda, NL). After 30 min of incubation, the MitoTracker-containing medium was replaced by fresh DMEM-glucose or DMEM-galactose and mitochondrial morphology was visualized with the EVOS® FL Color Imaging System (Life Technologies).

Caco-2 cell differentiation and polarisation

To induce differentiation and polarization, Caco-2 cells were seeded at a density of 1.0×10^5 cells/cm2 in 24-well transwell inserts (0.4 µM diameter ThinCertsTM, Greiner Bio-one, Alphen a/d Rijn, NL) and differentiated for 14 days in DMEM-glucose or DMEM-galactose.

Culture medium was changed three times a week and transepithelial electrical resistance (TEER) was monitored using a MilliCell-ERS voltohmmeter (Millipore, Etten-Leur, NL). Only the monolayers with TEER values exceeding 200 Ω × cm-2 were used for Caco-2 monolayer permeability experiments and for determination of OXPHOS protein levels and RT-qPCR gene expression analysis.

Western blotting to determine OXPHOS protein levels

14-days differentiated Caco-2 cells were washed with ice-cold HBSS, lysed and sonicated in 50 mM TRIS (pH 7.4) supplemented with 1% Triton (Sigma Aldrich) and 10% protease inhibitor (P8340, Sigma Aldrich). Experiments were performed in triplicate. Protein concentrations were determined with the Bio-Rad DC colorimetric protein assay (Bio-Rad laboratories, Veenendaal, NL) according to manufacturer's protocol. Protein samples were heated at 50°C for 5 min, separated (30 µg protein per sample per lane) by SDS-PAGE using 12% acrylamide gels and finally transferred to an Immobilon® transfer membrane (Millipore). Five individual proteins representing the five different OXPHOS complexes (NDUFB8 for Complex I; SDHB for Complex II; UQCRC2 for Complex III; COX II for Complex IV; ATP5A for Complex V) were detected (incubation overnight at 4°C) with total OXPHOS human antibody cocktail (1:1000, Ab110411, Abcam). Anti-beta-actin antibody (1:1000, Ab8227) was used as a loading control. Bound primary antibodies were visualized using IRDye 800CW goat anti-mouse IgG secondary antibody (1:10,000; LI-COR Biosciences Inc., Lincoln, NE, USA). Blots were scanned with LI-COR's Odyssey Infrared Image System.

Caco-2 monolayer permeability experiments

14-days differentiated Caco-2 cells were used for monolayer permeability experiments, which were all performed in triplicate. Caco-2 cells were incubated with varying concentrations of either vehicle (96% EtOH), CCCP (mitochondrial uncoupler, 0-1-2-3-4-5 µM), rotenone (ROT, Complex I inhibitor, 0-50-100-200 nM), and Piericidin A (PA, Complex I inhibitor, 0-50-100-200 nM; Cayman Chemicals, Ann Arbor, MI, USA) for 27 hours, 30 hours, and 42 hours, respectively. Changes in TEER were followed over time to determine permeability of the Caco-2 monolayer. Apical medium was harvested to determine cytotoxicity with the Pierce LDH Cytotoxicity assay kit (Thermo Scientific), according to the manufacturer's instructions.

100 ug/mL fluorescein (Sigma Aldrich) was added to the apical medium of Caco-2 cells incubated with vehicle (96% EtOH) or 200 nM PA for 24 hours prior to harvesting the basolateral medium at different time points. Caco-2 cells incubated with vehicle (96% EtOH) or 200 nM PA were also harvested at different time points to determine cellular energy status and to isolate RNA for gene expression analysis.

HPLC determination of cellular energy status of Caco-2 cells

14-days differentiated Caco-2 cells were incubated with vehicle (96% EtOH) or 200 nM PA and then harvested at several time points to measure cellular levels of ATP with high

pressure liquid chromatography analysis, adapted from Di Pierro et al. [16]. All samples were deproteinized by mixing with 100 µL 7% perchloric acid, 125 µL 1 M KOH and 75 µL 0.1 M phosphate buffer pH 6.5 followed by centrifugation for 10 min at 14000g at 4°C. Supernatant was snap-frozen in liquid nitrogen and stored at -80°C until further analysis. Twenty-five µL of the supernatant was analyzed on a HPLC system (LaChrom Elite, Merck-Hitachi) with UV detection at 254 nm (Kratos). The chromatography was performed on an HPLC C18 column (Aqua; 150×4.6 mm, 5 µm, Phenomenex) using a gradient elution with two mobile phases. Mobile phase A consisted of 0.1 M phosphate buffer pH 6.0 and mobile phase B consisted of 0.1 M phosphate buffer pH 6.0 / methanol (60/40). The gradient, at a flow rate of 1 mL/min, was as follows: 0-5 min isocratic at 0% mobile phase B; 5–16 min linear gradient from 0% to 22% B; 16–25 min linear gradient from 22% to 60% B; 25–27 min linear gradient from 60% to 100% B; 27-28 min isocratic at 100% B; 28-29 min linear return from 100% to 0% B; 29-35 min isocratic at 0% B, making a total run time of 35 min. Peaks were integrated with Clarity Lite software (DataApex, Prague, Czech Republic) and levels of cellular ATP were calculated using calibration curves made with commercially available standard (Sigma Aldrich).

RT-qPCR gene expression analysis

Total cellular RNA was isolated from 14-days differentiated Caco-2 cells incubated with vehicle (96% EtOH) or 200 nM PA at different time points using the Qiagen RNeasy Kit (Qiagen, Westburg, Leusden, NL) in combination with the on-column DNase I (Bio-Rad) treatment, according to the manufacturer's instructions. RNA quantity was checked with the Nanodrop spectrophotometer (IsoGen Life Science, Maarssen, NL) and RNA integrity was checked by capillary zone electrophoresis (Experion, Bio-Rad). RNA of all individual samples (n=6 per condition) was reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad). Gene expression measurements were performed in a 25 µL reaction mix containing iQ SYBR Green Supermix (Bio-Rad) using the CFX96 Real-Time System (Bio-Rad). Target and reference genes, gene symbols and primer sequences are shown in Table 6.1. Reference genes were selected based on stability. The expression of the gene of interest was normalized against the geometrical mean of the reference genes with the CFX software (Bio-Rad).

Statistics

The average values are presented as mean ± SD. All statistical analyses were performed in Graphpad Prism (Version 6.07). Student T-tests were applied to assess changes in OCR and the different OXPHOS complex protein levels between Caco-2 cells cultured in DMEM-glucose and those cultured in DMEM-galactose. Student T-tests were also applied to assess changes in LDH release, cellular ATP levels, fluorescein flux, and tight junction gene expression between Caco-2 cells treated with CCCP, PA or ROT and their corresponding controls. In case data were not normally distributed, data were log transformed (*TJP1*, *TJP2* and *CLDN2*). Gene expression of *TJP1* was also after log transformation not normally distributed and a Mann Withney U test was performed instead. One-way ANOVA with Sidak's multiple comparisons post-hoc testing was applied to assess differences in sucrase-

isomaltase gene expression between Caco-2 cells differentiated in DMEM-glucose and those differentiated in DMEM-galactose. Statistical significance was defined as a two-tailed $p < 0.05$.

Table 6.1. Primer sequences of reference genes and genes of interest

Reference genes	Forward primer 5'-3'	Reverse primer 5'-3'
Beta-actin (*ACTB*)	CTGGAACGGTGAAGGTGACA	AAGGGACTTCCTGTAACAATGCA
Beta-2-microglobulin (*B2M*)	TGCCGTGTGAACCATGTG	GCGGCATCTTCAAACCTC
Transmembrane protein 14C	CCGCTTGTTTTCTGCAGGTG	CACGCTGCCTGCTTTTACATAG
Glutaminyl-tRNA synthetase	TAAGTGACCTGAACCTGGCATC	GACGCTCAAACTGGAACTTGTC
Genes of interest	**Forward primer 5'-3'**	**Reverse primer 5'-3'**
Sucrase-isomaltase (*SIM*)	GAGGACACTGGCTTGGAGAC	ATCCAGCGGGTACAGAGATG
Tight junction protein 1 (*TJP1*)	GGGAACAACATACAGTGACGC	CCCCACTCTGAAAATGAGGA
Tight junction protein 2 (*TJP2*)	GGAGGATGTGCTTCATTCG	GGCCTCTTGACCACAATAG
Occludin (*OCLN*)	CCCATCTGACTATGTGGAAAGA	AAAACCGCTTGTCATTCACTTTG
Claudin 1 (*CLDN1*)	TTTCCTGCTACAACAATCCTCTCC	GTTGTTTTTCGGGGACAGGAAC
Claudin 2 (*CLDN2*)	CTCCCTGGCCTGCATTATCTC	ACCTGCTACCGCCACTCTGT
Claudin 7 (*CLDN7*)	CTGCAAAATGTACGACTCGGTG	GCAAGACCTGCCACGATGAAAA

Results

Galactose culturing switches Caco-2 cells towards a more oxidative metabolic phenotype

To establish the relation between mitochondrial ATP production and intestinal permeability it is essential that ATP production in the culture system is dependent on mitochondria and not on glycolysis only. This is not the case when Caco-2 cells are cultured in glucose-rich culture medium (data not shown). Therefore, we first investigated whether a switch to high-galactose (25mM) medium (DMEM-galactose) stimulates mitochondrial ATP production as described previously for other cell types [12].

Caco-2 cells show an increased density and an increase in branching of the mitochondrial network when grown on DMEM-galactose compared to DMEM-glucose (**Figure 6.1A**). In line with this finding, protein levels of subunits of the five different OXPHOS complexes were increased on DMEM-galactose compared to DMEM-glucose (**Figures 6.1B and C**). The largest increase was seen for complex I, followed by complex IV and II. Functionally, Caco-2 cells grown on DMEM-galactose showed increased basal and maximal OCR. These effects were similar for undifferentiated Caco-2 cells, as measured with the high-resolution oxygraph with undifferentiated cells (**Figure 6.1D**), and semi-differentiated Caco-2 cells, as measured with the Seahorse XF96 (**Figure 6.1E**).

Caco-2 cells differentiated on DMEM-galactose showed higher absolute TEER values (approximately 300 Ω·cm2 vs 200 Ω·cm2) and a higher increase in gene expression of the brush border enzyme sucrase-isomaltase (about 8-fold for glucose vs about 14-fold for galactose, **Figure 6.1F**), compared to DMEM-glucose. To provide a first link between

mitochondrial ATP production and Caco-2 monolayer permeability, indicated by TEER values, we uncoupled the mitochondria using CCCP, which is also used to determine maximal OCR. A dose-dependent decrease in TEER by CCCP was seen in 14-days differentiated Caco-2 cell monolayers when cultured in DMEM-galactose after 12 hours of incubation (**Figure 6.1G**). At 12 and 15 hours of CCCP incubation, LDH release was not increased indicating the absence of CCCP-induced cytotoxicity (**Figure 6.1H**).

Altogether, these findings indicate that Caco-2 cells cultured in DMEM-galactose switched to a more oxidative metabolic phenotype and became dependent on mitochondrial ATP production, which inhibition results in decreased Caco-2 monolayer permeability. Next to an effect on mitochondrial OXPHOS uncoupling, CCCP also may exert nonspecific effects in the cell [17, 18]. Therefore, we decided to continue the experiments with a different, more targeted method to obstruct mitochondrial ATP production by inhibiting complex I, which showed the largest increase in protein levels when Caco-2 culture medium was switched from DMEM-glucose to DMEM-galactose.

Rotenone and Piericidin A increase permeability and decrease cellular energy status of Caco-2 monolayer grown in DMEM-galactose

In the Caco-2 monolayers ROT induced, in a concentration-dependent manner, first an increase followed by a decrease in TEER when cultured in DMEM-galactose, but not DMEM-glucose (**Figure 6.2A**). However, ROT has been shown to have the potency to destabilize microtubules [19], which next to the actin cytoskeleton have been indicated to be critical in maintaining tight junctions and thus the integrity of the epithelial barrier [20]. These off-target effects have not been related to PA and therefore this Complex I inhibitor was used in the remainder of this study. Similar to ROT, PA induced in a concentration-dependent manner first an increase and then a decrease in TEER of Caco-2 monolayers cultured in DMEM-galactose, but not in DMEM-glucose (**Figure 6.2B**). The lowest concentration of PA of 50 nM was insufficient to induce a decrease in TEER, while 100 nM of PA resulted in a delayed but similar pattern as 200 nM of PA, which showed the largest effects on Caco-2 monolayer permeability without observed cytotoxicity (**Figure 6.2F**). It furthermore resulted in a significant reduction in the cellular energy status as reflected by decreased levels of ATP, already at the point where TEER was still increased, but more pronounced at the point where TEER was decreased (**Figure 6.2D**). The increase in Caco-2 monolayer permeability was also shown by an increased amount of fluorescein passing the monolayer (**Figure 6.2E**). These results suggest that an increase in Caco-2 monolayer permeability is a result from a decreased cellular energy status by inhibiting mitochondrial ATP production.
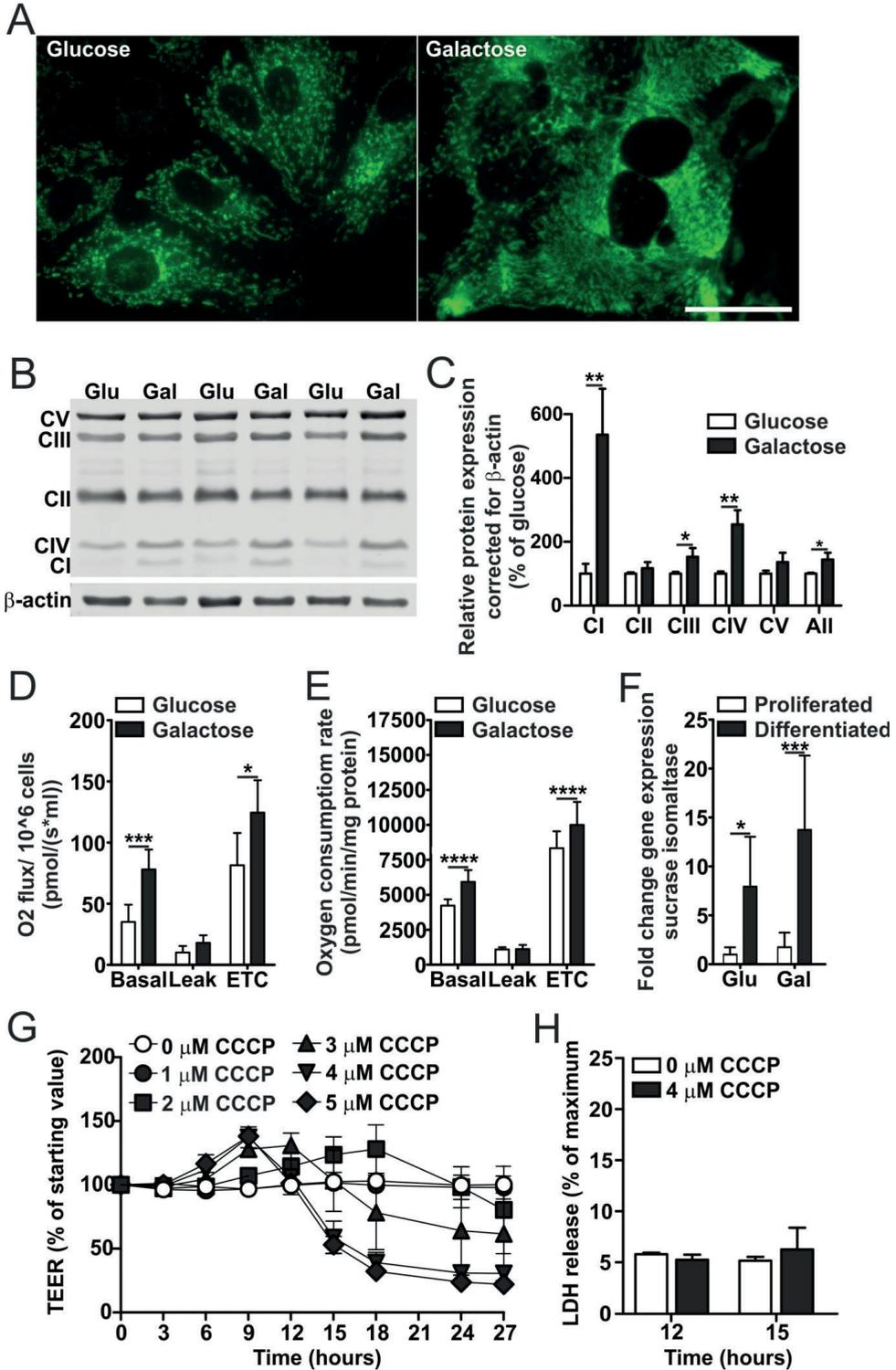
Figure 6.1. Oxidative metabolic characterization of Caco-2 cells grown on glucose and galactose substrate
Caco-2 cells were grown in DMEM-glucose and DMEM-galactose for 10 days, after which (A) mitochondrial structures, (B) and (C) levels of OXPHOS complexes, (D) basal, leak, and maximal oxygen consumption rates were determined. Caco-2 monolayers were semi-differentiated in DMEM-glucose and DMEM-galactose for 14 days after which (E) basal, leak, and maximal oxygen consumption rates were determined. 14-days differentiated Caco-2 monolayers grown in DMEM-glucose and DMEM-galactose were tested for (F) their sucrose-isomaltase gene expression; Gene expression was normalized with the mRNA gene expression of beta-actin and beta-2-microglobulin. The 14-days differentiated Caco-2 monolayers were incubated with CCCP (0-1-2-3-4-5 µM) after which (G) the transepithelial electrical resistance (TEER) and (H) the cytotoxicity as assessed by the release of lactate dehydrogenase (LDH) were followed over time. The results are expressed as a mean ± SD. The * symbol indicates statistical significance of $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$.
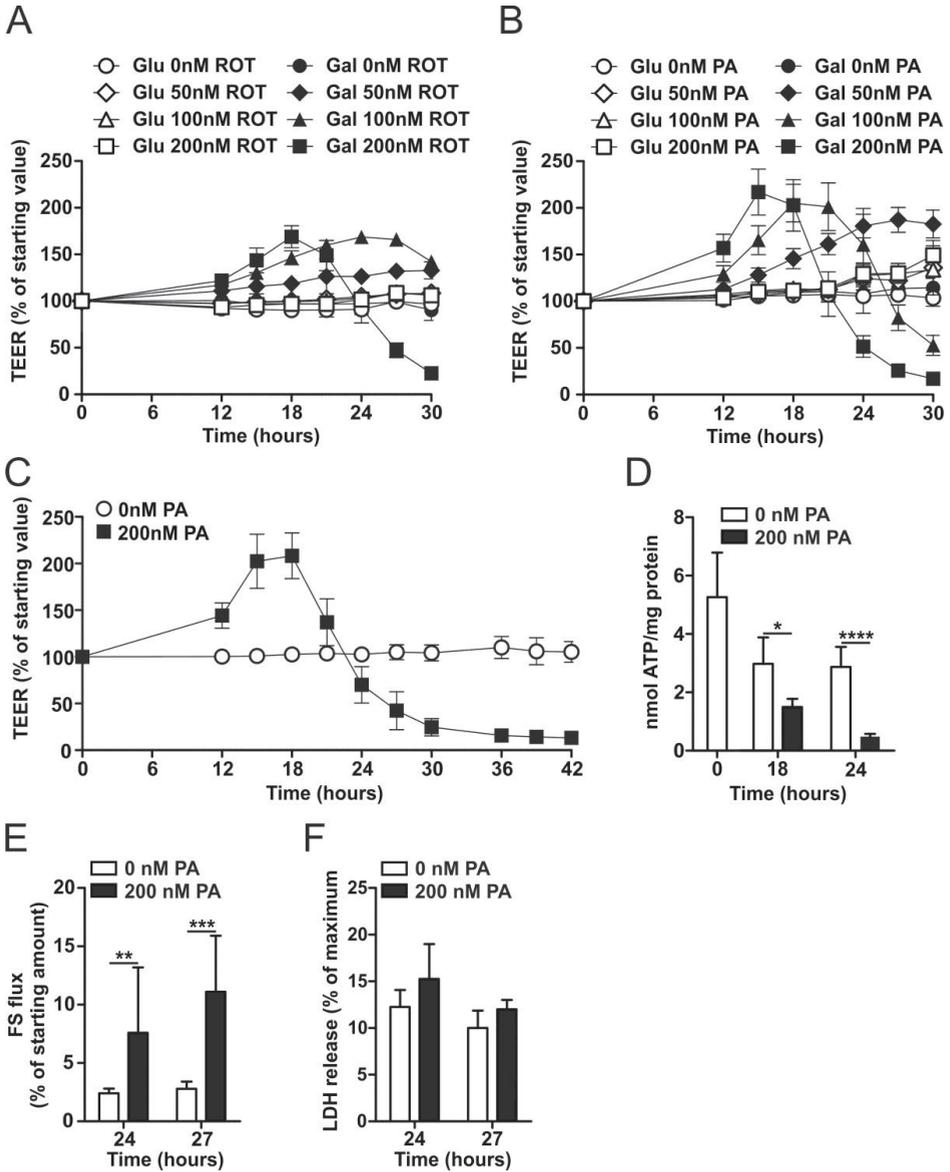
Figure 6.2. Effects OXPHOS Complex I inhibition on differentiated Caco-2 monolayers cultured in DMEM-glucose and DMEM-galactose

14-days differentiated Caco-2 monolayers were incubated with vehicle (96% EtOH) or rotenone (ROT; 50-100-200 nM) or Piericidin A (PA; 50-100-200 nM) after which TEER was followed over time, (A) and (B) show representative examples of a single experiment and (C) the average of three independent experiments. 14-days differentiated Caco-2 monolayers grown in DMEM-galactose were incubated with vehicle (96% EtOH) or 200 nM PA, after which (D) cellular ATP status, (E) fluorescein (FS) passage from the apical to the basolateral side of the monolayer, and (F) cytotoxicity by lactate dehydrogenase (LDH) release were determined. The results are expressed as a mean ± SD. The * symbol indicates statistical significance of $P < 0.05$, ** $P < 0.01$, and **** $P < 0.0001$.

Piericidin A alters the expression of tight junction genes in Caco-2 monolayers

mRNA gene expression analysis of tight junction genes was performed at the point of the clear increase (150-200%) and the clear decrease (40-60%) in TEER of the DMEM-galactose-cultured Caco-2 monolayers treated with 0 (control) or 200 nM PA. When the TEER was high, after approximately 15 hours of PA treatment (**Figure 6.2C**), the gene expression of tight junction protein 1 (*TJP1*; P = 0.04), occludin (*OCLN*; P = 0.001) and claudin 1 (*CLDN1*; P = 0.003) was significantly increased in Caco-2 cells treated with 200 nM PA as compared to control. In contrast, the gene expression of tight junction protein 2 (*TJP2*) was unchanged, while the expression of claudin 2 (*CLDN2*; P = 0.003) and claudin 7 (*CLDN7*; P = < 0.0001) were significantly decreased upon PA treatment compared to the control (**Figure 6.3**). When TEER was low, after approximately 24 hours of PA treatment (**Figure 6.2C**), these outcomes were more pronounced for *TJP1* (P = 0.002), *OCLN* (P = < 0.0001) and both *CLDN2* (P = < 0.0001) and *CLDN7* (P = < 0.0001). However, at this point the gene expression of *CLDN1* showed merely a trend (P = 0.06), while gene expression of *TJP2* became significantly increased (P = 0.02) upon PA treatment as compared to the control (**Figure 6.3**).
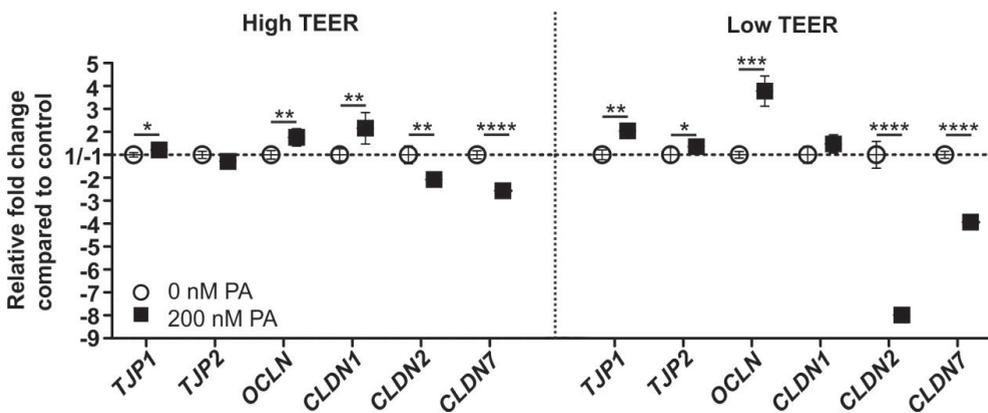

Figure 6.3. Gene expression of tight junctions in Caco-2 monolayers upon PA treatment
14-days differentiated Caco-2 monolayers grown in DMEM-galactose were incubated with vehicle (96% EtOH) or 200 nM Piericidin A (PA), after which mRNA gene expression of tight junction protein 1 (*TJP1*), tight junction protein 2 (*TJP2*), occludin (*OCLN*), claudin 1 (*CLDN1*), claudin 2 (*CLDN2*) and claudin 7 (*CLDN7*) between the moment when the transepithelial resistance (TEER) of the monolayer was high (High TEER) and low (Low TEER) were determined. Gene expression was normalized with the mRNA gene expression of the beta-2-microglobulin, transmembrane protein 14C, and glutaminyl-tRNA synthetase. The * symbol indicates statistical significance of P < 0.05, ** P < 0.01, *** P < 0.001, and **** P < 0.0001.

Discussion

In this study, we newly developed a Caco-2 cellular model that for its energy production was dependent on mitochondrial ATP production. To achieve this, a substrate switch from DMEM-glucose to DMEM-galactose was applied, based on previous experience with other

cell lines [12-15]. This model provides a situation more similar to the *in vivo* situation. Data from this model support that intestinal permeability is highly dependent on mitochondrial ATP production. Inducing mitochondrial dysfunction by either uncoupling or inhibition of OXPHOS function resulted in a decrease in cellular ATP levels, followed by a decrease in TEER and an increase in the paracellular flux of the fluorescein marker. This supports other studies that observed a relation between cellular ATP levels and cell monolayer permeability [21-25]. However, these models lack the representation of the *in vivo* situation, because they were cultured in high glucose medium. Caco-2 monolayers were subsequently incubated with 2-deoxyglucose, inhibiting glycolysis, or a combination of 2-deoxyglucose and antimycin A, inhibiting both glycolysis and OXPHOS, leading to a decrease in cellular ATP levels as well as an increase in monolayer permeability [24, 25]. Similar results were also found in Caco-2 cells treated with indomethacin, with or without ROT, both of which have been shown to inhibit OXPHOS Complex I [26]. The majority of these studies completely blocked glycolysis, OXPHOS (by inhibiting complex III), or both, which is a stringent inhibition of energy production. This is supported by the fact that effects were already observed within minutes. Importantly, very high levels (>200 times higher than levels used with our model) of OXPHOS inhibitors were used [25, 27], at doses that have been shown to induce show severe off-target effects that could lead to destabilization of microtubules [19], high oxidative stress [27], and Rho-GTPase-induced changes in tight junction or cytoskeleton structure [28, 29]. As ROT also showed off-target effects at very low concentrations, we included PA, which does not display these of target effects [28], and we decided to test the minimal concentration of ROT and PA that maximally reduced cellular ATP status and TEER, without apparent effects on cell damage/death. It can be concluded from the absence of any effect in the Caco-2 monolayers cultured in glucose-containing medium that both ROT and PA at these doses had no described off-target effects and that Caco-2 monolayers cultured in glucose-containing medium did not depend on their mitochondria for their ATP production.

Surprisingly, our studies showed first a dose-dependent increase in the TEER, followed by a similar dose-dependent decrease in TEER, independent of whether the Caco-2 monolayers were treated with CCCP, ROT, or PA. We speculate that since TEER is based on ion permeability of the monolayer, it may indicate a response of the cells that limits ion fluxes. The increase in TEER can therefore be suggested to reflect an initial defence mechanism of the intestinal epithelial cells to energy stress, whereby ATP-dependent ion channels, such Na+,K+-ATPase, are shutdown [25]. This may be part of a cellular energy conservation strategy since Na+,K+ATPase constitutes the major component of basal metabolic rate [1] and is fuelled by mitochondrial ATP [30]. Whether the observed increase in TEER would reflect an actual decrease in monolayer permeability for small molecules or only ion permeability cannot be assessed with the flux of fluorescein or labelled dextrans, as this measurement is only sensitive enough to determine an increase in monolayer permeability. Next, we aimed at obtaining a first insight in the underlying mechanism for these observed patterns in TEER by analysing the expression of several tight junction genes in Caco-2 monolayers incubated with PA. There are many proteins involved in the formation of the

tight junctions, serving various functions. The tight junction proteins together with OCLN mainly support and stabilize the structure of the tight junctions [31]. The observed increases in gene expression of *TJP1*, *TJP2*, and *OCLN* could be a compensatory mechanism of the Caco-2 cells, in an attempt to stabilize and protect the structure of the tight junctions [32]. In contrast, the claudins have shown to play an essential role in the actual paracellular permeability, by forming barriers that tighten or pores that open the paracellular pathway for selective molecules [33]. CLDN1 and CLDN7 have been mainly described to tighten the junctions by barrier formation [34-36], while CLDN2 opens the junction by pore formation [35, 37, 38]. The upregulation of *CLDN1* and downregulation of *CLDN2* could reflect a first defense mechanism to prevent an increase in intestinal permeability. Only the downregulation of the gene expression of 'tightening' *CLDN7* is not consistent with a protective response. It should be noted that changes in gene expression are inconclusive, since functionality will be determined by changes in levels and localisation of the different proteins. Indeed changes in tight junction protein localisation have been associated with monolayer permeability [39]. It has been shown that, in Caco-2, MDCK and glomerular epithelial cell monolayers, antimycin A-induced or ROT-induced ATP depletion altered the distribution of TJP1 and OCLN, without affecting their protein levels [21, 22, 40]. Since redistribution of these tight junction proteins affect their interactions with the cytoskeleton [23], these effects could possibly be caused by off-target effects rather than decreased ATP, but this remains to be elucidated.

The partial oxygen pressure of the small intestine is sufficient, but low. This renders the small intestine very sensitive to a decrease in supply of oxygen or an increase in demand with concommitent increase in infection and inflammation [41]. Depency on oxygen supply also explains the strong intestinal response to ischemia [2-4]. The small intestinal barrier is thus, at least partly, dependent on aerobic, i.e. mitochondrial energy production. This is not the case when the Caco-2 monolayers are grown in DMEM-glucose. Our DMEM-galactose Caco-2 model therefore provides a physiologically more relevant alternative. This oxidative Caco-2 model offers the possibility to study the mechanism underlying intestinal permeability as observed during *in vivo* situations.

This model could therefore be an important tool to unravel possible underlying mechanisms of intestinal permeability. Next to normal physiological conditions, such as during endurance exercise, intestinal permeability is also observed in many disease-related situations [42]. It is attractive to think that by studying intestinall permeability in detail using this Caco-2 model could potentially offer therapeutic targets to improve disease states.

Conclusion

In conclusion, mitochondria play an essential role in maintaining an intestinal barrier with high integrity. This is supported by the dependency on mitochondrial ATP production of Caco-2 monolayers grown on DMEM-galactose, which is mimicking the *in vivo* situation by showing a more oxidative metabolic phenotype that is responsive to mitochondrial dysfunction.

Conflict of interest

The authors declare that they have no conflict of interest.

References

1. Rolfe, D. and G.C. Brown, Cellular energy utilization and molecular origin of standard metabolic rate in mammals. Physiological Reviews, 1997. 77(3): p. 731-758.
2. Hinnebusch, B.F., et al., Enterocyte response to ischemia is dependent on differentiation state. Journal of Gastrointestinal Surgery, 2002. 6(3): p. 403-409.
3. Grootjans, J., et al., Human intestinal ischemia-reperfusion–induced inflammation characterized: Experiences from a new translational model. The American journal of Pathology, 2010. 176(5): p. 2283-2291.
4. Grotz, M., et al., Intestinal cytokine response after gut ischemia: role of gut barrier failure. Annals of Surgery, 1999. 229(4): p. 478.
5. Van Wijck, K., et al., Exercise-induced splanchnic hypoperfusion results in gut dysfunction in healthy men. PLoS ONE, 2011. 6(7): p. e22366.
6. Rodenburg, W., et al., Impaired barrier function by dietary fructo-oligosaccharides (FOS) in rats is accompanied by increased colonic mitochondrial gene expression. BMC Genomics, 2008. 9(1): p. 1.
7. Somasundaram, S., et al., Uncoupling of intestinal mitochondrial oxidative phosphorylation and inhibition of cyclooxygenase are required for the development of NSAID-enteropathy in the rat. Alimentary Pharmacology and Therapeutics, 2000. 14(5): p. 639.
8. Mahmud, T., et al., Nonsteroidal antiinflammatory drugs and uncoupling of mitochondrial oxidative phosphorylation. Arthritis & Rheumatism, 1996. 39(12): p. 1998-2003.
9. Bjarnason, I. and K. Takeuchi, Intestinal permeability in the pathogenesis of NSAID-induced enteropathy. Journal of Gastroenterology, 2009. 44(19): p. 23-29.
10. Sambuy, Y., et al., The Caco-2 cell line as a model of the intestinal barrier: influence of cell and culture-related factors on Caco-2 cell functional characteristics. Cell Biology and Toxicology, 2005. 21(1): p. 1-26.
11. Yee, S., In vitro permeability across Caco-2 cells (colonic) can predict in vivo (small intestinal) absorption in man—fact or myth. Pharmaceutical Research, 1997. 14(6): p. 763-766.
12. Rossignol, R., et al., Energy substrate modulates mitochondrial structure and oxidative capacity in cancer cells. Cancer research, 2004. 64(3): p. 985-993.
13. Kase, E.T., et al., Remodeling of oxidative energy metabolism by galactose improves glucose handling and metabolic switching in human skeletal muscle cells. PLoS ONE, 2013. 8(4).
14. Aguer, C., et al., Galactose enhances oxidative metabolism and reveals mitochondrial dysfunction in human primary muscle cells. PLoS ONE, 2011. 6(12): p. e28536-e28536.
15. Marroquin, L.D., et al., Circumventing the Crabtree effect: replacing media glucose with galactose increases susceptibility of HepG2 cells to mitochondrial toxicants. Toxicological sciences, 2007. 97(2): p. 539-547.
16. Di Pierro, D., et al., An Ion-Pairing High-Performance Liquid Chromatographic Method for the Direct Simultaneous Determination of Nucleotides, Deoxynucleotides, Nicotinic Coenzymes, Oxypurines, Nucleosides, and Bases in Perchloric Acid Cell Extracts. Analytical Biochemistry, 1995. 231(2): p. 407-412.
17. Gottlieb, E., et al., Mitochondrial membrane potential regulates matrix configuration and cytochrome c release during apoptosis. Cell Death & Differentiation, 2003. 10(6): p. 709-717.
18. Lichtshtein, D., H.R. Kaback, and A.J. Blume, Use of a lipophilic cation for determination of membrane potential in neuroblastoma-glioma hybrid cell suspensions. Proceedings of the National Academy of Sciences, 1979. 76(2): p. 650-654.
19. Marshall, L.E. and R.H. Himes, Rotenone inhibition of tubulin self-assembly. Biochimica et Biophysica Acta (BBA) - General Subjects, 1978. 543(4): p. 590-594.
20. Glotfelty, L.G., et al., Microtubules are required for efficient epithelial tight junction homeostasis and restoration. American Journal of Physiology - Cell Physiology, 2014. 307(3): p. C245-C254.
21. Wagner, M.C., et al., Ischemic Injury to Kidney Induces Glomerular Podocyte Effacement and Dissociation of Slit Diaphragm Proteins Neph1 and ZO-1. The Journal of Biological Chemistry, 2008. 283(51): p. 35579-35589.

22. Gopalakrishnan, S., et al., aPKC-PAR complex dysfunction and tight junction disassembly in renal epithelial cells during ATP depletion. American Journal of Physiology - Cell Physiology, 2007. 292(3): p. C1094-C1102.
23. Tsukamoto, T. and S.K. Nigam, Tight junction proteins form large complexes and associate with the cytoskeleton in an ATP depletion model for reversible junction assembly. Journal of Biological Chemistry, 1997. 272(26): p. 16133-16139.
24. Unno, N., et al., Hyperpermeability and ATP depletion induced by chronic hypoxia or glycolytic inhibition in Caco-2BBe monolayers. American Journal of Physiology - Gastrointestinal and Liver Physiology, 1996. 270(6): p. G1010-G1021.
25. Bacallao, R., et al., ATP depletion: a novel method to study junctional properties in epithelial tissues. I. Rearrangement of the actin cytoskeleton. Journal of Cell Science, 1994. 107(12): p. 3301-3313.
26. Carrasco-Pozo, C., M. Gotteland, and H. Speisky, Apple peel polyphenol extract protects against indomethacin-induced damage in Caco-2 cells by preventing mitochondrial complex I inhibition. Journal of Agricultural and Food Chemistry, 2011. 59(21): p. 11501-11508.
27. Carrasco-Pozo, C., et al., Differential protective effects of quercetin, resveratrol, rutin and epigallocatechin gallate against mitochondrial dysfunction induced by indomethacin in Caco-2 cells. Chemico-Biological Interactions, 2012. 195(3): p. 199-205.
28. Grefte, S., et al., Rotenone inhibits primary murine myotube formation via Raf-1 and ROCK2. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research, 2015. 1853(7): p. 1606-1614.
29. Sanchez, M., et al., Rotenone-induced toxicity is mediated by Rho-GTPases in hippocampal neurons. Toxicological Sciences, 2008. 104(2): p. 352-361.
30. Fernández-Moncada, I. and L.F. Barros, Non-preferential fuelling of the Na+/K+-ATPase pump. Biochemical Journal, 2014. 460(3): p. 353-361.
31. Van Itallie, C.M., et al., ZO-1 stabilizes the tight junction solute barrier through coupling to the perijunctional cytoskeleton. Molecular Biology of the Cell, 2009. 20(17): p. 3930-3940.
32. Zeissig, S., et al., Changes in expression and distribution of claudin 2, 5 and 8 lead to discontinuous tight junctions and barrier dysfunction in active Crohn's disease. Gut, 2007. 56.
33. Lee, S.H., Intestinal permeability regulation by tight junction: implication on inflammatory bowel diseases. Intestinal Research, 2015. 13(1): p. 11-18.
34. Tanaka, H., et al., Intestinal deletion of Claudin-7 enhances paracellular organic solute flux and initiates colonic inflammation in mice. Gut, 2015. 64(10): p. 1529-1538.
35. Hou, J., et al., Study of claudin function by RNA interference. Journal of Biological Chemistry, 2006. 281(47): p. 36117-36123.
36. Alexandre, M.D., Q. Lu, and Y.-H. Chen, Overexpression of claudin-7 decreases the paracellular Cl– conductance and increases the paracellular Na+ conductance in LLC-PK1 cells. Journal of Cell Science, 2005. 118(12): p. 2683-2693.
37. Amasheh, S., et al., Claudin-2 expression induces cation-selective channels in tight junctions of epithelial cells. Journal of Cell Science, 2002. 115(24): p. 4969-4976.
38. Rosenthal, R., et al., Claudin-2, a component of the tight junction, forms a paracellular water channel. Journal of Cell Science, 2010. 123(11): p. 1913-1921.
39. Price, D.B., et al., Peanut Allergens Alter Intestinal Barrier Permeability and Tight Junction Localisation in Caco-2 Cell Cultures. Cellular Physiology and Biochemistry, 2014. 33(6): p. 1758-1777.
40. Carrasco-Pozo, C., P. Morales, and M. Gotteland, Polyphenols protect the epithelial barrier function of Caco-2 cells exposed to indomethacin through the modulation of occludin and zonula occludens-1 expression. Journal of agricultural and food chemistry, 2013. 61(22): p. 5291-5297.
41. Zeitouni, N.E., et al., The impact of hypoxia on intestinal epithelial cell functions: consequences for invasion by bacterial pathogens. Molecular and cellular pediatrics, 2016. 3(1): p. 1.
42. Bjarnason, I., A. Macpherson, and D. Hollander, Intestinal permeability: an overview. Gastroenterology, 1995. 108(5): p. 1566-1581.

Chapter 7

General Discussion

Aims

To better understand intestinal permeability towards proteins, the first aim of the research presented in this thesis was to examine the effect of a widely experienced physiological challenge, exercise, on the permeability of the intestine to dietary proteins and peptides. In proof-of-concept studies, the intestinal permeability for small inert sugars was compared with that for dietary proteins and peptides, obtained from 2 different dietary sources. The effect of exercise on permeability and on a variety of systemic responses related to the intestine were studied.

The second aim was to determine the role of oxidative metabolism in maintenance of intestinal epithelial integrity. This information could contribute to explain *in vivo* observations and is a first step towards the development of a physiologically relevant human *in vitro* intestinal epithelial model to study intestinal permeability.

This last chapter will reflect on the main outcomes of the previous chapters. Next, some strengths and weaknesses of the studies performed will be placed in the broader context of methodological considerations for future study designs. A hypothesis will be given for the mechanism of exercise-induced increase in intestinal permeability. Implications of the findings and directions for future research in intestinal permeability will also be given. Finally, some general conclusions will be drawn.

Main findings

- Endurance exercise induced intestinal permeability to inert sugar molecules.
- The casein-derived peptide betacasomorphin-7 crossed the intestinal barrier to a greater extent following exercise, as compared to rest
- The peanut-derived allergen Ara h 6 crossed the intestinal epithelial barrier to a greater extent when consumption was followed by endurance exercise as compared to rest, which correlated well with the increased uptake of lactulose.
- Replacing the energy substrate glucose by galactose induced a switch to a more aerobic phenotype in Caco-2 cells, which made them susceptible to mitochondrial dysfunction.
- Mitochondrial dysfunction in galactose-cultured Caco-2 monolayers leads to ATP depletion and an increase in intestinal permeability.

Lessons learned from *in vivo* studies - Methodological considerations for future design of studies.

Exercise as a challenge to increase intestinal permeability

We know from literature that endurance exercise is able to induce an increase in intestinal permeability towards inert sugar markers. Most studies focused on the effect of running and cycling. Exercise-induced permeability is measured with the paracellular passage of an inert disaccharide such as lactulose. This is, however, a relatively small macromolecule and the question remained to which extent larger macromolecules such as dietary proteins and peptides are also able to cross the intestinal barrier following exercise. Food-Dependent Exercise-Induced Anaphylaxis (FDEIA) is a rare condition in which individuals only respond to the allergic food when ingested in combination with exercise [1, 2] or low doses of aspirin [3, 4]. This suggests that exercise can indeed lead to an increased intestinal permeability to dietary proteins. Although the assumption is often made, evidence from studies in humans on the uptake of intact protein without exercise is scarce and incomplete [5], even less information is available in combination with exercise. Husby and colleagues [6-8] and Paganelli and colleagues [9, 10] showed in several studies that allergens can be detected in their intact form in the blood after food consumption in healthy individuals, and it was shown that coeliac disease may lead to increased uptake of these allergens, which supports that intestinal permeability could be an underlying cause. It has been suggested that more of an antigen leaks into the circulation upon acute exercise, but this was seen in sensitised mice and not in unsensitised mice [11]. These measurements were indirect as no analyses were performed in serum and exercise-induced permeability was not determined. In contrast, Matsuo and colleagues [12] did show an increase in circulating levels of dietary (gliadin) peptides during exercise as compared to rest, also in healthy individuals. However, they did not assess intestinal permeability and therefore could not speculate on possible routes of uptake.

In my thesis research, I performed two proof-of-concept studies that provided the evidence that endurance exercise indeed results in an increased amount of dietary protein and peptides in the circulation, coinciding with an increase in intestinal permeability as measured with several markers. With the studies performed in this thesis, we cannot conclude which mechanism accounted most for the increase in intestinal permeability to peanut allergen: increased transcellular transport or decreased intestinal integrity due to changes in tight junctions (increasing paracellular transport) or loss of epithelial cells. On the one hand, the fact that the increase in peanut allergen uptake was highly correlated with the increase in the uptake of the paracellular marker lactulose suggests that increased uptake of dietary proteins and peptides during exercise is mediated via paracellular transport. On the other hand, a decrease in cell integrity may also be involved as the increase in peanut allergen uptake also correlated with the release of fatty acid binding protein-2 (FABP2), a direct marker for epithelial cell damage which will be discussed in more detail later. FABP2,

however, also correlated well with the uptake of the sugars, which makes it difficult to interpret what is actually directly or indirectly related to each other.

"Intestinal permeability:
The ability of compounds to cross the intestinal epithelial barrier."

Definition in thesis:
The ability of compounds to cross the intestinal epithelial barrier

Intestinal permeability

Transcellular transport

Intactness barrier = intestinal integrity

Cell integrity

Tight junctions

Paracellular transport

See Figure 1.2
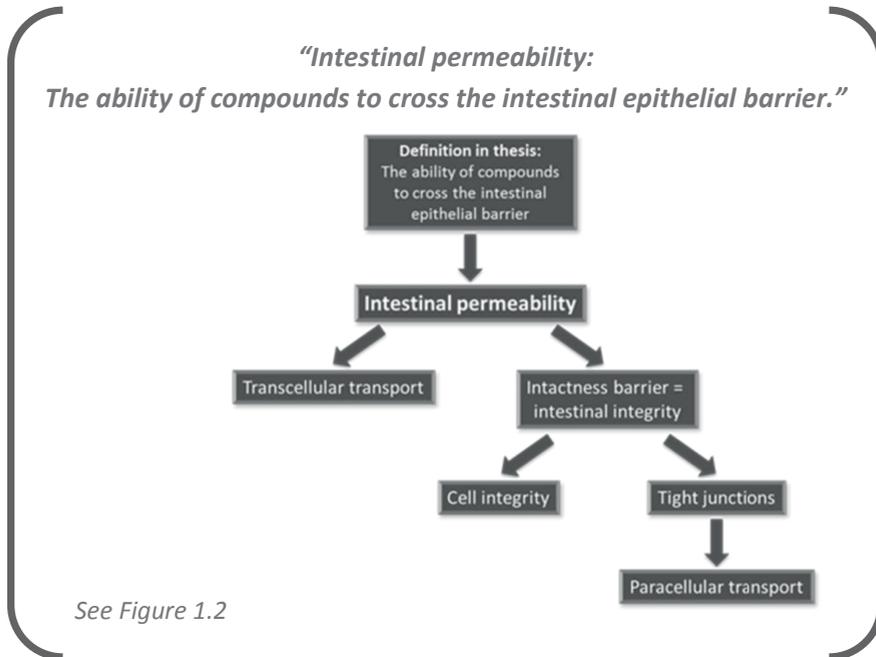
In any case, exercise seems to compromise intestinal integrity and thereby increase intestinal permeability to dietary proteins. It has been shown *in vitro* that peanut allergens are able to cross human intestinal Caco-2 cell monolayers [13]. The peanut allergens altered tight junction protein localisation in Caco-2 monolayers, which possibly facilitated uptake via the paracellular route. To confirm whether this holds true also *in vivo*, animal models in combination with exercise challenges could be used in which the small intestine can be harvested and studied in more detail for its permeability as well as morphology and tight junction protein localisation. Next to this, small intestine-specific knockout and transgenic animals could be developed to study the role of specific factors in intestinal permeability. A system that combines Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) with Cas 9, an RNA-guided DNA cleaving enzyme, can be used to develop human cell models and animal models with tissue-specific DNA edits. This very recently discovered and developed system is a breakthrough in genome engineering, offering a relatively efficient way to very specifically edit genomes and adjust gene regulation [14]. Much of this technology has been developed and reviewed by the laboratories of Doudna [14-16] and van der Oost [17, 18]. By blocking or activating processes involved in transcellular transport, intestinal integrity, or mitochondrial oxidative metabolism, their potential role in exercise-induced intestinal permeability can be studied in more detail.

The sugar test as a marker for intestinal permeability

It is important to choose the right method to assess intestinal permeability [19]. In human studies, intestinal permeability is measured by the intestinal epithelial passage of at least two marker molecules into the blood or urine. As already discussed in the general introduction, several markers have been proposed, and most often the lactulose/rhamnose or the lactulose/mannitol dual sugar test is applied, which are currently considered to be the golden standard in clinical practice [19].

In contrast to certain disease models, in which intestinal permeability is often chronically increased, there is only a short window to measure an increase in intestinal permeability induced by a physiological challenge such as endurance exercise. Consequences of exercise may be also less apparent than disease-induced permeability. Therefore, it is important that changes in intestinal permeability with the sugar test can be detected with high sensitivity. In this regard, several factors are important in the study design of exercise interventions:

First, the timing of the ingestion of the sugar test solution is an important factor that can affect outcome. In literature, different timings of consumption of the sugar solution have been applied; before, during, and after the onset of exercise. During our first study (Protégé Study, chapters 2 and 3), the participants were instructed to ingest the sugar solution immediately after they finished the endurance exercise. The measured changes in intestinal permeability were not as large as expected and in fact, the lactulose/rhamnose ratio was increased due to lower uptake of rhamnose rather than increased uptake of lactulose. Several explanations can be suggested for the limited exercise-induced permeability. First, the participants were highly trained and their intestine may have been adapted to the exercise-induced stress. Second, the endurance exercise of 50% Wmax may not have been intensive enough to elicit the expected response. Although this level of exercise was perceived by the participants as being very heavy in combination with the glycogen depletion exercise that was done as part of the protocol on the previous evening, it may have not been strenuous enough to induce effects on intestinal permeability. A third explanation could be that the glycogen depletion exercise may have had a protective pre-conditioning effect on the endurance exercise. Unfortunately, the effect of each of the two exercise phases within the dual exercise intervention were not assessed separately, to obtain clarity. Finally, permeability may be only increased during exercise and be restored shortly thereafter. This explanation is in line with the concept that oxygen and nutrient drainage towards muscle and away from the intestine induces permeability [20].

During the second study (PEANUTS Study, chapter 5), the participants were instructed to ingest the sugar solution at the start of the exercise protocol. In this setting we found a clear exercise-induced increase in intestinal permeability for each of the participants. Although several factors were different from the first study, such as untrained individuals instead of highly trained individuals and a single exercise intervention at 70% Wmax instead of a dual exercise intervention ending with 50% Wmax, we believe that the timing of the intake of the sugar solution may have played a major role in finding much more pronounced effects in

exercise-induced permeability. When we look at direct effects of exercise on the small intestine, the marker of small intestinal epithelial damage, fatty acid binding protein-2 (FABP2) appears to be released into the blood stream mainly during the exercise and its levels drop rapidly when the exercise is finished [20-22]. Directly after the exercise has finished, the recovery phase starts, likely also rapidly attenuating the exercise-induced permeability. This strengthens the notion that permeability needs to be detected during the challenge, in this case exercise, and that it is more difficult to detect intestinal permeability when the sugar test is applied directly after the exercise rather than just before or during the exercise.

A second factor to consider in the application of the sugar test is at which time point after consumption and in which biological fluid the sugars should be analysed. During the first study we measured the sugar levels in both a plasma sample taken after 1 and 5 h urine. Due to practical feasibility, urine was pooled over a period of 5 hours, but the analysis of the plasma taken after 1 h showed to be a more sensitive indicator for the exercise-induced changes in uptake of the sugars [20, 21]. Collecting urine at set time points proved to be more difficult than expected, but 5 hours of urine collection may have been too long, as it has been shown that the sugar test in 2 h urine is more sensitive than in 5 h urine [23]. Overall, even though slightly more invasive with regard to sample collection, we would recommend the detection of the sugar levels in plasma over detection in urine. If urine is used, then it seems best to limit the time window of collection to 2 hours.

A third factor to consider in the application of the sugar test is the validity of the markers. Even though used frequently, the inertness of lactulose in the small intestine is under debate. Lactulose is fermented by the microbiota in the large intestine and therefore only reflects permeability of the small intestine [24]. Fermentation of lactulose, however, could also take place in the small intestine. Although this is generally considered only to be of importance in case of small intestinal bacterial overgrowth, for which the lactulose breath test has been developed for detection [25], also the small intestine of healthy individuals contains a considerable amount of microbiota able to ferment carbohydrates [26]. Inter-individual differences in microbiota can possibly lead to a large variation in outcome, decreasing the power for statistics. This is why it is important that the sugar test contains a monosaccharide, such as rhamnose or mannitol, as a reference of which the uptake is unaffected by changes in intestinal permeability, but is similarly affected as lactulose by other potential modulatory factors such as bacterial degradation. If the treatment affects these modulatory factors, the ratio of the two sugars gives a more reliable measure of intestinal permeability than when lactulose is used alone [27]. The drawback of rhamnose and mannitol in the analysis is that these monosaccharides are often also detected in significant levels in plasma and urine at baseline, before the sugar solution is ingested, possibly due to their rather ubiquitous presence in food [28]. This challenges the sensitivity of the dual sugar test. It also resulted in the exclusion of one participant in the statistical

analysis of the PEANUTS Study described in chapter 5, which is highly undesirable. However, at this moment no better alternative is available. In next studies in which the sugar test is applied, it would be recommended to also standardize the meal(s) before the study test day, to minimalize high pre-ingestion levels.

In general, the sugar test is a good way to functionally determine intestinal permeability, but standardized protocols are needed to be able to compare different studies with each other, a notion that is also emphasized in a recent review by Galipeau and Verdu [19].

Biomarkers of intestinal integrity

Next to changes in intestinal permeability measured by alteration of sugar transport, several biomarkers have been proposed that reflect barrier integrity, which could also influence intestinal permeability. Two of such biomarkers are citrulline [29] and fatty acid binding protein-2 (FABP2)[30], which were included in our studies.

FABP2 is considered a useful marker for the direct effects of an intervention on intestinal epithelial integrity [30-32]. The primary function of FABP2, however, is in the facilitation of intracellular fatty acid uptake and it has been studied in particular in relation to type 2 diabetes [33]. The reason to consider this cytoplasmic protein as a biomarker of acute compromise in intestinal integrity is because i) it is exclusively and highly expressed in the small intestine [34], ii) it is a small (15kDa) protein relatively rapidly diffusing from the damaged epithelial cell into the blood stream when the intestinal epithelial cell is damaged [30], and iii) the half-life of this enzyme is relatively short (approximately 11 minutes) [35]. Many studies have indeed shown high specificity for FABP2 with small intestinal villus atrophy [30].

In our studies, however, FABP2 proved to be rather sensitive to mild stress situations as levels were relatively high at baseline compared to exercising values when participants arrived to the research setting on bicycle in fasted state (chapters 2 and 5). These high levels at baseline question the clinical relevance of using this protein as a biomarker for intestinal integrity with exercise challenges, mainly because endurance exercise had a minimal additive effect compared to the baseline values, at which point intestinal injury should be considered minimal. Next to this, not all participants were responders for FABP2, also shown by van Wijck et al. [36]. Taken this knowledge together, we would advise others to critically consider whether FABP2 is a sensitive enough and clinically relevant marker of exercise-induced changes in intestinal integrity. If it is nevertheless decided to use FABP2, then it is important to let the participants rest sufficiently after arrival at the research setting and after insertion of the catheter. A rest of at least half an hour to become more relaxed is advised so that more reliable baseline values for FABP2 are obtained and small exercise-induced effects may be more easily detected.

Citrulline is an interesting biomarker for lower enterocyte mass because the small intestine is the major source of citrulline production from glutamine, it is not incorporated in proteins, and release and uptake of citrulline by the liver are negligible [37]. In intestinal diseases with villous atrophy, such as Celiac Disease, or when individuals have less enterocyte mass due to a short bowel syndrome or partial resection of the small intestine, fasted citrulline levels in the blood have been shown to be lower than in healthy controls [29], but Peter et al. [38] indicated that a citrulline generation test, which consists of a challenge test with the intake of 20 grams of alanine-glutamine, could be a more sensitive tool to detect villous atrophy. These authors showed that fasted levels of citrulline did not differ, but the slope and iAUC of plasma citrulline after the alanine-glutamine challenge was much lower in patients than in healthy controls [38]. During the Protégé study similar observations were made after the intake of 40 grams of casein protein (chapter 2). When the protein was ingested directly after exercise, the increase in plasma citrulline levels was largely absent compared to the increase that is seen when the protein was ingested in rest [21]. It can be debated, however, whether levels of citrulline should also be considered a useful marker for acute exercise-induced villous atrophy. In contrast, we hypothesized that lower plasma levels of citrulline after protein intake reflect lower enterocyte metabolic activity or capacity due to the energy depletion in the intestine during and directly after exercise rather than a decrease in absolute enterocyte mass due to villous atrophy [21]. For chronic conditions, citrulline may be a good marker for enterocyte mass, but for acute physical challenges such as endurance exercise, the use of systemic levels of citrulline may be of limited value in relation to intestinal integrity.

Design of exercise challenges in human intervention studies

There are at least three factors that should be taken into account when designing a human intervention trial in which an exercise challenge is included to induce intestinal permeability. Those will be discussed shortly below.

First, the exercise protocol should be simple and effective. To this end, cycling is an endurance type of exercise which can be relatively easy standardized in a research setting and is therefore often chosen as an exercise intervention. My thesis research indicated that the exercise protocol does not need to be complicated to be effective. Even though it could have been partly the result of better timing of the sugar test, as discussed earlier, the simple protocol of one hour of cycling at 70% in less trained individuals resulted in a clear exercise-induced increase in intestinal permeability, while the more complex dual cycling intervention with an overnight step in highly trained individuals, which were needed to complete the protocol, did not show such clear results.

Second, it is preferred to have an exercise challenge that is as well relevant as feasible in different populations. It would be preferred to choose an exercise challenge which most different populations are able to complete. Cycling for one hour at 70% of their maximal workload is feasible for a group of healthy young individuals, but much less for a group of elderly or patients. It should be studied whether cycling at a lower workload and/or shorter

time would also result in relevant increases in intestinal permeability in different populations. In elderly, inducing intestinal permeability with an exercise challenge may be more difficult, because with a reduction in muscle mass and much lower workload, the reduction in splanchnic blood has been shown to be less pronounced [39].

Third, there are some factors to consider to standardize and control the intervention trial as much as possible. It is known from literature that the gut is 'trainable' when it comes to the ingestion of carbohydrates during and after exercise [40, 41], but not so much is known when it comes to the adaptation to exercise-induced permeability. Within the Protégé study, I determined the test-retest repeatability and found that intestinal permeability showed adaptation already upon a single retesting. This resulted in several hitches regarding the repeatability of the study and interpretation of the outcomes. It would be interesting to find out whether adaptation is also seen when a single exercise challenge is repeated, at different workloads, in different populations and with different intervals. Next, there could be any first-time-effect in highly trained individuals, which would suggest the need for a training session and possibly also strict standardization of exercise allowed to be performed by the participants in a certain time period prior to the study period.

Intestinal permeability- The relation with energy metabolism

Recently, the correlation between increased intestinal permeability and disease has caught the attention of the public and popular media, who persistently illustrate the leaky gut as an all-embracing cause of multiple diseases. This has led to a rise in acceptance of the diagnosis of a "leaky gut syndrome" and although so far not yet demonstrated, supporters of this diagnosis argue that repair of the intestinal epithelial barrier would cure underlying diseases. There are many different processes that are related to intestinal permeability and the function of the intestine in digestion and absorption of protein, as studied in this thesis, is only one of those processes.

It would be fascinating to find out whether the observed increase in transport of dietary proteins induced by exercise corresponds with the transport of these proteins in specific intestinal diseases in which an increase in intestinal permeability is seen. This would substantiate the use of an exercise challenge in humans as a model to study and strengthen intestinal permeability in disease conditions.

To support this at the mechanistic level, it would be also interesting to understand how induction of intestinal permeability by endurance exercise relates to mechanistic causes of intestinal permeability as observed in different diseases, such as Inflammatory Bowel Disease. In both cases, the deprivation of oxygen could be a causative factor, although induced in different ways.

A lower supply of oxygen affects especially the intestine, as this organ is particularly sensitive to changes in oxygen supply. This is because the intestinal epithelium exists in a state of relative 'physiological hypoxia', already under normal physiological conditions [42-44]. Therefore small changes in oxygen supply can already lead to oxygen deprivation, which in

turn can result in mitochondrial ATP depletion. The hypoxic state of the intestinal epithelial cells is followed by an increase in intestinal permeability [45, 46].

Exercise-induced blood redistribution is the most accepted hypothesis for the underlying mechanism of exercise-induced permeability [36, 47]. With this redistribution, more blood is directed to the muscles, while less blood is directed to the splanchnic organs, which results in a lower supply of oxygen and energy substrates. In Inflammatory Bowel Disease or other intestinal inflammatory diseases, on the other hand, an oxygen deficit can result from increased infiltration of innate immune cells, which also consume a considerable amount of oxygen [48]. Either way, both processes, exercise-induced changes in blood flow or increased oxygen demand due to inflammation, could result in an oxygen deficit. Together with a lower availability of energy substrate, this could lead to an energy-depleted state of the intestinal epithelial cells, which we hypothesize induces an increase in intestinal permeability.

The process towards increased intestinal permeability is evidently a multifactorial one, but part of the mechanisms could be similar. In any case, more mechanistic studies have to be performed to elucidate the exact mechanism of intestinal permeability and how these can be affected by different physiological conditions. In my thesis research I used an *in vitro* Caco-2 cell model to study the mechanism of intestinal permeability and this will be discussed next.

Lessons learned from *in vitro* studies - Methodological considerations for future study design

In vitro Caco-2 model to study intestinal permeability

In vivo human trials are a good way to characterise intestinal permeability in the context of the complete host system. It does not, however, offer many possibilities to study underlying mechanisms, unless biopsies are taken, but those are rather invasive and provide only snap-shot information. Animal models with specific alterations in processes related to intestinal permeability, whether or not in combination with exercise, provide the possibility to investigate mechanisms while maintaining physiological complexity. However, as is the case for human studies, animal studies can be complex and time-consuming.

Cell studies provide a further alternative. Although they lack the physiological complexity of the whole organism, cell models offer a high level of control and specificity which renders them very suitable for mechanistic studies and screening purposes.

In this thesis, monolayers of the human intestinal cell line Caco-2 were used to examine the mechanism underlying exercise-induced permeability. In chapter 6 it was shown that mitochondrial inhibition resulted in increased permeability, which would substantiate a role for decreased oxygen supply in increased permeability as suggested in the previous paragraph. Chapter 6 also describes adaptations to the Caco-2 cell model which have been made to obtain a more *in vivo*-like situation to study intestinal permeability. Caco-2

monolayers that are grown on galactose medium switched to a more aerobic phenotype than those grown on glucose medium. This corroborates the *in vivo* situation, where intestinal cells are thought to depend on oxidative metabolism, based on high vascularisation and sensitivity for ischemia damage and ischemia/reperfusion damage [49].

It would be interesting, and highly relevant, to compare the aerobic phenotype of cultured Caco-2 cells and human enterocytes, sampled by biopting, by analysis of mitochondrial respiration with respirometry (Oroboros or Seahorse) and by examining related mRNA and protein levels with qPCR and Western blot, respectively.

Further improvement of the Caco-2 cell model developed during my PhD research could be made with regard to culturing conditions. One aspect is oxygen availability, which is much higher in routine culture than it actually is *in vivo*. The partial oxygen pressure *in vivo* at the serosal (basolateral) side of the intestinal epithelial cells lies around 60 mmHg [43, 50], while this is only half at the luminal (apical) side [43]. Studies with Caco-2 monolayers have shown that with routine culture, the partial oxygen pressure of the basolateral medium was around 110 mmHg, which is almost double as seen in the *in vivo* situation [51]. The apical medium was not directly measured, but could be estimated to fluctuate around 50 mmHg, so also double as seen in the *in vivo* situation. It would be interesting to study the effect of lowering the supplied oxygen from 160 mmHg to a level that results in levels more similar to *in vivo* oxygen levels, but without inducing real hypoxia, on the aerobic phenotype of the Caco-2 cell model and how this relates to intestinal permeability.

An important aspect that is missing in the Caco-2 model developed in this thesis is the continuous mucus layer, which covers the intestinal epithelial cell monolayer and plays an essential role in intestinal permeability [52, 53]. Co-culture models with differentiated Caco-2 intestinal epithelial cells and differentiated HT29-MTX mucus-producing goblet cells have therefore been suggested and developed, especially to study intestinal permeability to drugs and peptides [54, 55].

There are furthermore several easily overlooked, but potentially essential factors that have to be taken into account during the study of intestinal permeability of Caco-2 monolayers. First is the large difference in effects that can be observed between proliferating Caco-2 cells and differentiated Caco-2 cells. First experiments on the effects of the complex I inhibitor rotenone on cellular ATP status were performed with proliferating Caco-2 cells, for which 24 hours of treatment with levels of 20 nM was sufficient to induce cellular ATP depletion without affecting cell viability. In later experiments with differentiated Caco-2 monolayers, however, stimulation with rotenone did not show to have any significant effect on cellular energy status nor monolayer permeability unless the concentration was increased at least 5-10 fold to 100-200 nM . Rotenone treatment with these high levels of rotenone, however, has major effects on cell viability of proliferating Caco-2 cells. This learned us that outcomes of titration experiments in proliferating Caco-2 cells are not always representative for differentiated Caco-2 monolayers. One explanation could be that energetic demands are

much higher during proliferation, and possibly also during differentiation, than during maintenance of the differentiated state of the Caco-2 cells monolayers. In our group, we more often observed that fully differentiated monolayers are much less responsive to different types of stimuli than those in earlier phases of monolayer differentiation (unpublished date). That is one reason to choose for a 14-day differentiation period for these experiments rather than the frequently used 21-day differentiation period, especially when Caco-2 cells are grown on galactose, because galactose seems to stimulate spontaneous Caco-2 differentiation.

Another factor is the discrepancy in effects that can be observed with apical and basolateral treatment of Caco-2 cell monolayers. We observed large differences in one-sided treatment of Caco-2 monolayers with the complex inhibitors. Treating these monolayers with 200 nM of rotenone or Piericidin A on the apical side resulted in much lower responses than when applied on the basolateral side. One explanation could be that the apical membrane of the epithelial cells have more defence mechanisms than the basolateral membrane, because *in vivo* the apical membrane is also the first coming into contact with these compounds. Possible defence mechanisms include efflux pumps and ABC transporters [56, 57], to pump out potentially harmful substances. ABC transporters such as P-glycoprotein and breast cancer resistance protein are expressed on the apical membrane of the intestinal epithelial cells and can prevent the absorption of compounds into the body [58]. Furthermore, P-glycoprotein- (Mdr1a-) deficient mice showed serious gastrointestinal problems, including spontaneous colitis and severe inflammation [59].

Implications and directions for future research

My PhD project was part of the 'IPOP Customized Nutrition research programme', a programme that focused on global protein supply and the use of novel proteins in food and feed. This program arose from the prediction by the Food and Agriculture Organization of the United Nations (FAO) that the demand for animal-sourced foods will almost double between 2000 and 2030 due to the fast growth of the global population and changes in consumption patterns with an increase in urbanization in several parts of the world [60]. Within the 'IPOP Customized Nutrition research programme', knowledge was obtained to support possible solutions which may close the upcoming gap between the demand and supply of protein. In this quest, there is a strong focus on novel proteins derived from various sources such as plants, algae, food waste, and insects [61-63]. Next to the technical criteria, questions that arise from the field of novel proteins are directed at their potential effects on animal and human health, with respect to their digestibility and absorption [63], and their potency to activate the immune system [64]. This safety aspect is of particular importance as I have shown in chapters 2 and 5 of this thesis that changes in intestinal barrier function (e.g. increased permeability) under different physiological conditions can result in increased absorption of large molecules including protein fragments [21], which can

potentially induce an immune response. In my thesis I present possible ways to study intestinal permeability and underlying mechanisms, and the last part of this discussion I would like to dedicate to key steps that should be taken to test novel proteins with regard to digestion and absorption and implications of the models developed in my thesis research.

The critical and perhaps most difficult step in the characterization of digestion and absorption is the detection of proteins and derived peptides that arise after digestion and absorption. Depending on the goal, different techniques are available, such as liquid chromatography-tandem mass spectrometry and enzyme-linked immunosorbent assay, with or without prior immunoprecipitation. It proves to be difficult to detect dietary peptides in the circulation and there can be several explanations for this.

First, the levels of absorbed dietary proteins and peptides are very low, as reported in chapter 4 and 5 a peak level corresponding to about 0.0001% of the ingested peanut protein could be detected in plasma, and detection methods therefore need a very high specificity and sensitivity to correctly detect a dietary protein or derived peptide of interest, which is still a challenge [65, 66].

Second, although an educated guess can be made based on *in vitro* experiments, it is often not exactly known which proteins and peptides are formed upon digestion *in vivo*. Heterogeneity in individual digestibility in combination with modifying factors, such as physiological or pathophysiological conditions, makes it very difficult to pinpoint on which protein or derived peptide should be focussed with regard to absorption into the circulation. As a starting point it could be a good idea to focus on peptides that are resistant to digestion *in vitro*. This is not only practical, but may be relevant as well, since bioactive peptides and allergens are indeed resistant to digestion [67]. Detection methods can then be developed that specifically target such peptides, as was reported here for Ara h 6 in chapter 4. If mass spectrometry is applied, the use of intrinsically labelled proteins could support the detection of small peptides with higher specificity in humans [68].

Lastly, in chapter 4 it has also been described how endogenous immunoglobulin G (IgG) antibodies can interfere with an ELISA detection method, as the endogenous IgG antibody potentially blocks the binding epitope for the antibody used in the detection assay. This could truly underestimate the actual amount of the protein or peptide that is able to cross the intestinal epithelial barrier, and cause large variations in outcome between individuals. It is not yet clear against which peptides and protein we have these IgGs formed and what it exactly means. It has been stated that the levels of protein-specific IgG are not a valuable tool for diagnostics in the field of food allergies [69], but several studies do describe symptom improvement for migraine and IBD when they apply a diet in which foods are eliminated against which the patients have high levels of IgG [70-72]. With regard to the use and study of novel proteins, these IgGs should be factors taken into account.

To find out which digestion-resistant peptides are formed after digestion of novel proteins, a good *in vitro* digestion model is needed. This model needs to be reproducible and relevant to the *in vivo* situation. A harmonized protocol for the digestion of proteins has been developed within the INFOGEST COST action, in order to set up an *in vivo* relevant model for protein digestion [73], which could make future research better comparable with the use of the same protocol. The reproducibility in digestion outcomes of this harmonized protocol showed promising results compared to the digestion outcomes of protocols applied by different laboratories [74]. To increase its *in vivo* relevance, a more complex dynamic digestion model could be applied [75]. The model could possibly be further adapted by including the action of the brush border enzymes. This can be achieved by adding an incubation step with a solution of rat brush border peptidases, which is commercially available. This step is redundant when *in vitro* digestion is followed by an *in vitro* absorption model in which the brush border peptidases are included, such as a Caco-2 intestinal epithelial cell model.

Once potential digestion-resistant peptides are determined and can be detected with high specificity and sensitivity, the next step in assessing the safety of novel food proteins is to study the digestion and absorption kinetics of these novel proteins *in vitro* and *in vivo*.
The more physiologically relevant Caco-2 model described in chapter 6 could serve as an improved *in vitro* model to study intestinal permeability to novel proteins and derived peptides. This model can be further improved by implementing physiological oxygen conditions and increasing the cell layer complexity to mimic the intestine, for example, by co-culturing with mucus-secreting cells. Alternatively, other techniques, such as the ex vivo intestinal segment model, the Ussing chamber technique, or intestinal epithelial organoids can be used to mimic *in vivo* absorption [76-78].

A next approach could be to study the digestion and absorption of novel proteins or derived peptides in vivo. In my thesis I presented a human intervention study in which an exercise challenge was included to study the effect of physiological challenges on intestinal permeability to dietary proteins and peptides (chapters 2 and 5). Several important aspects to consider when designing such intervention studies have been addressed in more detail in earlier parts of this discussion. This human intervention study, whether or not in combination with an exercise challenge, can be applied to study intestinal permeability to a broad range of novel proteins, provided that adequate detection methods for these proteins or derived peptides are developed. This human intervention study with exercise challenge can be used in future research in several directions. First, it would be highly interesting to study the underlying mechanisms of intestinal permeability on cellular level *in vivo*. Although pretty challenging with regard to practical feasibility, combining the exercise challenge with a small intestinal biopsy would offer the possibility to study *in vivo* the effect of exercise on morphology, tight junction protein levels and localization, and cellular metabolism. The

described human intervention with exercise challenge is also ideal to screen for the effects of a wide range of dietary compounds, so not only novel proteins, for improvement of intestinal permeability. The exercise challenge may show beneficial or deleterious effects of these dietary compounds which may not be detected when intestinal permeability is determined only in rest. This challenge furthermore fits very well in the new nutrition and health research field of 'resilience' or 'phenotypic flexibility' [79, 80].

Conclusions

Overall, the observations as outlined in this thesis provide the evidence that the intestine is permeable to dietary proteins and peptides and that a widely experienced physiological challenge, exercise, enhances this permeability (chapters 2-5). Although correlations with several markers for intestinal permeability are indicated, we need to be careful to assign this observation to one or more of the potential causal factors: increased transcellular transport, decreased cellular integrity, or decreased intestinal barrier integrity (increased paracellular transport). More mechanistic studies are needed to pinpoint the underlying mechanism of increased intestinal permeability to dietary proteins and peptides following exercise. These mechanistic insights are crucial for correct interpretation of the data.

Chapter 6 provides evidence that mitochondrial energy production may play an essential role in the maintenance of intestinal integrity. This mechanism could play an important role in exercise-induced intestinal permeability. A physiologically more relevant model has been developed to study intestinal permeability and underlying mechanisms, and future research could further improve and validate this model.

Finally, the totality of the research presented in this thesis provides tools to study the potential health effects of novel proteins with regard to digestion and absorption, which is of importance in the search for alternative protein sources to close the predicted gap between global protein supply and demand.

References

1. Loibl, M., et al., Definition of an exercise intensity threshold in a challenge test to diagnose food-dependent exercise induced anaphylaxis. Allergy, 2009. 64.
2. Hanakawa, Y., et al., Food-dependent exercise-induced anaphylaxis:a case related to the amount of food allergen ingested. British Journal of Dermatology, 1998. 138.
3. Fujii, H., et al., Food-Dependent Exercise-Induced Anaphylaxis Induced by Low Dose Aspirin Therapy. Allergology International, 2008. 57(1): p. 97-98.
4. Harada, S., et al., Aspirin enhances the induction of type I allergic symptoms when combined with food and exercise in patients with food-dependent exercise-induced anaphylaxis. British Journal of Dermatology, 2001. 145.
5. Reitsma, M., et al., Protein transport across the small intestine in food allergy. Molecular Nutrition & Food Research, 2014. 58(1): p. 194-205.
6. Husby, S., et al., Passage of dietary antigens into the blood of children with coeliac disease. Quantification and size distribution of absorbed antigens. Gut, 1987. 28(9): p. 1062-1072.
7. Husby, S., J.C. Jensenius, and S.E. SVEHAG, Passage of undegraded dietary antigen into the blood of healthy adults Further characterization of the kinetics of uptake and the size distribution of the antigen. Scandinavian Journal of Immunology, 1986. 24(4): p. 447-455.
8. Husby, S., J.C. Jensenius, and S.E. Svehag, Passage of Undegraded Dietary Antigen into the Blood of Healthy Adults. Scandinavian Journal of Immunology, 1985. 22(1): p. 83-92.
9. Paganelli, R. and R. Levinsky, Solid phase radioimmunoassay for detection of circulating food protein antigens in human serum. Journal of Immunological Methods, 1980. 37(3): p. 333-341.
10. Paganelli, R., et al., Immune complexes containing food proteins in normal and atopic subjects after oral challenge and effect of sodium cromoglycate on antigen absorption. Lancet, 1979. 313(8129): p. 1270-1272.
11. Yano, H., Y. Kato, and T. Matsuda, Acute exercise induces gastrointestinal leakage of allergen in lysozyme-sensitized mice. European Journal of Applied Physiology, 2002. 87(4): p. 358-364.
12. Matsuo, H., et al., Exercise and aspirin increase levels of circulating gliadin peptides in patients with wheat-dependent exercise-induced anaphylaxis. Clinical & Experimental Allergy, 2005. 35(4): p. 461-466.
13. Price, D.B., et al., Peanut Allergens Alter Intestinal Barrier Permeability and Tight Junction Localisation in Caco-2 Cell Cultures. Cellular Physiology and Biochemistry, 2014. 33(6): p. 1758-1777.
14. Sternberg, Samuel H. and Jennifer A. Doudna, Expanding the Biologist's Toolkit with CRISPR-Cas9. Molecular Cell, 2015. 58(4): p. 568-574.
15. Doudna, J.A. and E.J. Sontheimer, The Use of CRISPR/cas9, ZFNs, TALENs in Generating Site Specific Genome Alterations. Vol. 546. 2014: Academic Press.
16. Doudna, J.A. and E. Charpentier, The new frontier of genome engineering with CRISPR-Cas9. Science, 2014. 346(6213): p. 1258096.
17. Brouns, S.J.J., et al., Small CRISPR RNAs Guide Antiviral Defense in Prokaryotes. Science, 2008. 321(5891): p. 960-964.
18. van der Oost, J., et al., Unravelling the structural and mechanistic basis of CRISPR-Cas systems. Nature Reviews Microbiology, 2014. 12(7): p. 479-492.
19. Galipeau, H.J. and E.F. Verdu, The complex task of measuring intestinal permeability in basic and clinical science. Neurogastroenterology & Motility, 2016. 28(7): p. 957-965.
20. Van Wijck, K., et al., Exercise-induced splanchnic hypoperfusion results in gut dysfunction in healthy men. PLoS ONE, 2011. 6(7): p. e22366.
21. JanssenDuijghuijsen, L.M., et al., The effect of endurance exercise on intestinal integrity in well-trained healthy men. Physiological Reports, 2016. 4(20): e12994.
22. Van Wijck, K., et al., Aggravation of exercise-induced intestinal injury by Ibuprofen in athletes. Medicine and Science in Sports and Exercise, 2012. 44(12): p. 2257-2262.

23. Van Nieuwenhoven, M., et al., The sensitivity of the lactulose/rhamnose gut permeability test. European Journal of Clinical Investigation, 1999. 29(2): p. 160-165.

24. Meddings, J.B. and I. Gibbons, Discrimination of site-specific alterations in gastrointestinal permeability in the rat. Gastroenterology, 1998. 114(1): p. 83-92.

25. Riordan, S.M., et al., The lactulose breath hydrogen test and small intestinal bacterial overgrowth. The American Journal of Gastroenterology, 1996. 91(9).

26. Zoetendal, E.G., et al., The human small intestinal microbiota is driven by rapid uptake and conversion of simple carbohydrates. 2012. 6(7): p. 1415-1426.

27. Bjarnason, I., A. Macpherson, and D. Hollander, Intestinal permeability: an overview. Gastroenterology, 1995. 108(5): p. 1566-1581.

28. Grover, M., et al., 13C mannitol as a novel biomarker for measurement of intestinal permeability. Neurogastroenterology & Motility, 2016.

29. Crenn, P., et al., Postabsorptive plasma citrulline concentration is a marker of absorptive enterocyte mass and intestinal failure in humans. Gastroenterology, 2000. 119.

30. Pelsers, M.M.A.L., W.T. Hermens, and J.F.C. Glatz, Fatty acid-binding proteins as plasma markers of tissue injury. Clinica Chimica Acta, 2005. 352(1–2): p. 15-35.

31. Derikx, J.P., et al., Detection of chemotherapy-induced enterocyte toxicity with circulating intestinal fatty acid binding protein. Journal of Pediatric Hematology/Oncology, 2006. 28.

32. Niewold, T.A., M. Meinen, and J. van der Meulen, Plasma intestinal fatty acid binding protein (I-FABP) concentrations increase following intestinal ischemia in pigs. Research in Veterinary Science, 2004. 77(1): p. 89-91.

33. Vogel Hertzel, A. and D.A. Bernlohr, The Mammalian Fatty Acid-binding Protein Multigene Family: Molecular and Genetic Insights into Function. Trends in Endocrinology & Metabolism, 2000. 11(5): p. 175-180.

34. Shields, H.M., et al., Light microscopic immunocytochemical localization of hepatic and intestinal types of fatty acid-binding proteins in rat small intestine. Journal of Lipid Research, 1986. 27(5): p. 549-557.

35. van de Poll, M.C., et al., Liver manipulation causes hepatocyte injury and precedes systemic inflammation in patients undergoing liver resection. World Journal of Surgery, 2007. 31(10): p. 2033-2038.

36. van Wijck, K., et al., Physiology and pathophysiology of splanchnic hypoperfusion and intestinal injury during exercise: strategies for evaluation and prevention. American Journal of Physiology - Gastrointestinal and Liver Physiology, 2012. 303(2): p. G155-G168.

37. Windmueller, H.G. and A.E. Spaeth, Source and fate of circulating citrulline. American Journal of Physiology - Endocrinology And Metabolism, 1981. 241(6): p. E473-E480.

38. Peters, J., et al., The citrulline generation test: proposal for a new enterocyte function test. Alimentary Pharmacology & Therapeutics, 2008. 27(12): p. 1300-1310.

39. Kenney, W.L. and C. Ho, Age alters regional distribution of blood flow during moderate-intensity exercise. Journal of Applied Physiology, 1995. 79(4): p. 1112-1119.

40. Jeukendrup, A. and J. McLaughlin, Carbohydrate ingestion during exercise: effects on performance, training adaptations and trainability of the gut, in Sports Nutrition: More Than Just Calories - Triggers for Adaptation, R.J. Maughan and L.M. Burke, Editors. 2011, Karger: Basel. p. 1-17.

41. Cox, G.R., et al., Daily training with high carbohydrate availability increases exogenous carbohydrate oxidation during endurance cycling. Journal of Applied Physiology, 2010. 109(1): p. 126-134.

42. Chun, C., L. Zheng, and S.P. Colgan, Tissue metabolism and host-microbial interactions in the intestinal mucosa. Free Radical Biology and Medicine.

43. Zeitouni, N.E., et al., The impact of hypoxia on intestinal epithelial cell functions: consequences for invasion by bacterial pathogens. Molecular and cellular pediatrics, 2016. 3(1): p. 1.

44. Fisher, E.M., et al., Noninvasive monitoring of small intestinal oxygen in a rat model of chronic mesenteric ischemia. Cell Biochemistry and Biophysics, 2013. 67(2): p. 451-459.

45. Derikx, J.P., et al., Rapid reversal of human intestinal ischemia-reperfusion induced damage by shedding of injured enterocytes and reepithelialisation. PLoS ONE, 2008. 3(10): p. e3428.

46. Ohri, S.K., et al., The effect of intestinal hypoperfusion on intestinal absorption and permeability during cardiopulmonary bypass. Gastroenterology, 1994. 106: p. 318-318.

47. Otte, J.A., et al., Exercise induces gastric ischemia in healthy volunteers: a tonometry study. Journal of Applied Physiology, 2001. 91(2): p. 866-871.

48. Campbell, Eric L., et al., Transmigrating Neutrophils Shape the Mucosal Microenvironment through Localized Oxygen Depletion to Influence Resolution of Inflammation. Immunity, 2014. 40(1): p. 66-77.

49. Rosero, O., et al., Impaired intestinal mucosal barrier upon ischemia-reperfusion:"patching holes in the shield with a simple surgical method". BioMed Research International, 2014. 2014.

50. Thermann, M., et al., Oxygen supply of the human small intestine in mechanical ileus. Langenbecks Archiv fur Chirurgie, 1984. 363(3): p. 179-184.

51. Zeitouni, N.E., et al., Measuring oxygen levels in Caco-2 cultures. Hypoxia, 2015. 3: p. 53.

52. Rupani, B., et al., Relationship between disruption of the unstirred mucus layer and intestinal restitution in loss of gut barrier function after trauma hemorrhagic shock. Surgery, 2007. 141(4): p. 481-489.

53. Boshi, Y., et al., Developmental Changes in Distribution of the Mucous Gel Layer and Intestinal Permeability in Rat Small Intestine. Journal of Parenteral and Enteral Nutrition, 1996. 20(6): p. 406-411.

54. Hilgendorf, C., et al., Caco-2 versus caco-2/HT29-MTX co-cultured cell lines: Permeabilities via diffusion, inside- and outside-directed carrier-mediated transport. Journal of Pharmaceutical Sciences, 2000. 89(1): p. 63-75.

55. Wikman-Larhed, A. and P. Artursson, Co-cultures of human intestinal goblet (HT29-H) and absorptive (Caco-2) cells for studies of drug and peptide absorption. European Journal of Pharmaceutical Sciences, 1995. 3(3): p. 171-183.

56. Mao, Q., Role of the breast cancer resistance protein (ABCG2) in drug transport. The AAPS Journal, 2005. 7(1): p. E118-E133.

57. Omae, T., et al., Transient up-regulation of P-glycoprotein reduces tacrolimus absorption after ischemia–reperfusion injury in rat ileum. Biochemical Pharmacology, 2005. 69(4): p. 561-568.

58. Huls, M., F.G.M. Russel, and R. Masereeuw, The Role of ATP Binding Cassette Transporters in Tissue Defense and Organ Regeneration. Journal of Pharmacology and Experimental Therapeutics, 2009. 328(1): p. 3-9.

59. Resta-Lenert, S., J. Smitham, and K.E. Barrett, Epithelial dysfunction associated with the development of colitis in conventionally housed mdr1a–/– mice. American Journal of Physiology - Gastrointestinal and Liver Physiology, 2005. 289(1): p. G153-G162.

60. Robinson, T. and F. Pozzi, Mapping supply and demand for animal-source foods to 2030. Animal Production and Health Working Paper, 2011. 2.

61. Aiking, H., Future protein supply. Trends in Food Science & Technology, 2011. 22(2–3): p. 112-120.

62. Smil, V., Worldwide transformation of diets, burdens of meat production and opportunities for novel food proteins. Enzyme and Microbial Technology, 2002. 30(3): p. 305-311.

63. Boland, M.J., et al., The future supply of animal-derived protein for human consumption. Trends in food science & technology, 2013. 29(1): p. 62-73.

64. Ladics, G., et al., Review of animal models designed to predict the potential allergenicity of novel proteins in genetically modified crops. Regulatory Toxicology and Pharmacology, 2010. 56(2): p. 212-224.

65. Michalski, A., J. Cox, and M. Mann, More than 100,000 detectable peptide species elute in single shotgun proteomics runs but the majority is inaccessible to data-dependent LC– MS/MS. Journal of Proteome Research, 2011. 10(4): p. 1785-1793.

66. Hortin, G.L., The MALDI-TOF mass spectrometric view of the plasma proteome and peptidome. Clinical Chemistry, 2006. 52(7): p. 1223-1237.

67. Hartmann, R., et al., Cytotoxic and Allergenic Potential of Bioactive Proteins and Peptides. Current Pharmaceutical Design, 2007. 13(9): p. 897-920.

68. Van Loon, L., et al., The production of intrinsically labeled milk protein provides a functional tool for human nutrition research. Journal of Dairy Science, 2009. 92(10): p. 4812-4822.

69. Stapel, S.O., et al., Testing for IgG4 against foods is not recommended as a diagnostic tool: EAACI Task Force Report. Allergy, 2008. 63.

70. Alpay, K., et al., Diet restriction in migraine, based on IgG against foods: A clinical double-blind, randomised, cross-over trial. Cephalalgia, 2010. 30(7): p. 829-837.
71. Zar, S., et al., Food-specific IgG4 antibody-guided exclusion diet improves symptoms and rectal compliance in irritable bowel syndrome. Scandinavian Journal of Gastroenterology, 2005. 40.
72. Atkinson, W., et al., Food elimination based on IgG antibodies in irritable bowel syndrome: a randomised controlled trial. Gut, 2004. 53.
73. Minekus, M., et al., A standardised static *in vitro* digestion method suitable for food–an international consensus. Food & Function, 2014. 5(6): p. 1113-1124.
74. Egger, L., et al., The harmonized INFOGEST *in vitro* digestion method: From knowledge to action. Food Research International, 2015. 88, Part B: p. 217-225.
75. Dupont, D. and A.R. Mackie, Static and dynamic *in vitro* digestion models to study proteins stability in the gastrointestinal tract. Drug Discovery Today: Disease Models, 2016.
76. Ripken, D. and H. Hendriks, Porcine Ex Vivo intestinal segment model, in COST action FA 1005 "The Impact of Food Bioactives on Health", K. Verhoeckx, et al., Editors. 2015, Springer. p. 255-262.
77. Westerhout, J., H. Wortelboer, and K. Verhoeckx, Ussing Chamber, in COST action FA 1005 "The Impact of Food Bioactives on Health", K. Verhoeckx, et al., Editors. 2015, Springer. p. 263-273.
78. Lukovac, S. and G. Roeselers, Intestinal crypt organoids as experimental models, in COST action FA 1005 "The Impact of Food Bioactives on Health", K. Verhoeckx, et al., Editors. 2015, Springer. p. 245-253.
79. van Ommen, B., et al., Phenotypic flexibility as key factor in the human nutrition and health relationship. Genes & Nutrition, 2014. 9(5): p. 423.
80. Stroeve, J.H.M., et al., Phenotypic flexibility as a measure of health: the optimal nutritional stress response test. Genes & Nutrition, 2015. 10(3): p. 13.

Summary

The gut plays a pivotal role in human wellbeing by forming the body's largest barrier between the external and internal environment. It has a major function in the digestion and absorption of nutrients and at the same time protects the body from the uptake of potentially harmful compounds. An increase in intestinal permeability could affect the uptake of larger fragments of food components such as partly undigested dietary proteins. Endurance exercise is a widely experienced physiological challenge known to increase intestinal permeability, but the subsequent effects on protein uptake are less studied. Furthermore, several underlying mechanisms for exercise-induced permeability have been hypothesized, but have not been substantiated.

The aim of this thesis was to examine the effect of exercise intestinal permeability to dietary protein and peptides. Several *in vivo* proof-of-concept studies have been performed to study the effect of exercise on intestinal permeability towards small inert sugars versus small (casein-derived betacasomorphin-7) and large (peanut-derived allergen Ara h 6) dietary peptides. An *in vitro* model was developed to study one of the possible underlying mechanisms for an increase in intestinal permeability.

Chapter 1 provides background on intestinal integrity and permeability and their role in digestion and absorption of dietary protein. Furthermore, the *in vivo* and *in vitro* models for intestinal permeability as applied in the next chapters are introduced. Additionally, the research aim and the outline of this thesis are presented.

Chapters 2, 3, 4, and 5 describe *in vivo* findings on intestinal permeability. Chapters 2 and 3 show findings of the Protégé study; this study consisted of 12 well-trained healthy young men who consumed a casein protein solution in rest or directly after completing a strenuous dual exercise protocol.
Chapter 2 shows the effect of the strenuous exercise on urinary peptide excretion and markers of intestinal function and recovery, and inflammation. The exercise protocol resulted in increased urinary excretion of the casein-derived peptide betacasomorphin-7, even though intestinal permeability as measured with inert sugars was less pronounced. The exercise protocol also resulted in major changes in post-prandial amino acid profiles in plasma. It was concluded that strenuous exercise could have an effect on the amount of food-derived (bioactive) peptides crossing the epithelial barrier. In chapter 3 the test-retest repeatability of the outcomes of this strenuous exercise intervention is studied, since the complete intervention was performed twice in two separate weeks. It was shown that the metabolic effect parameters, such as glucose, lactate, and energy expenditure showed low test-retest variation, while stress response parameters to the exercise, such as creatine kinase, fibroblast growth factor 21 and intestinal permeability as measured with a lactulose/rhamnose challenge test showed high test-retest variation. It was concluded that even in well-trained young men an adapted response can be seen in exercise-induced stress after only a single repetition of the exercise intervention. This finding has implications for the design of human studies aiming at evaluating physiological responses to exercise.

Summary

In chapters 4 and 5, we focused on peanut protein instead of casein and compared to the Protégé study less trained individuals were included. These individuals reflect the general population level of training.

Chapter 4 describes the PEANUTS Pilot study, which was performed to develop an ELISA method to detect the major peanut allergen Ara h 6 in serum after peanut consumption. This protein could not be detected in four out of ten tested individuals. It was shown with spiking experiments that high levels of Ara h 6-specific immunoglobulin G in the blood hampered the detection of this allergen. This may be a broader phenomenon in studies on the uptake of food allergens in the circulation, and may explain why variable levels of food allergen in serum have been reported in literature. In chapter 5 the developed ELISA detection method from chapter 4 is used in the PEANUTS Study. Potential participants were first screened for the ability to detect Ara h 6 in their serum by a spiking experiment. The PEANUTS study consisted of 10 untrained healthy young men and women who consumed a lactulose/rhamnose test solution and 100 grams of mildly-roasted unsalted peanuts. The first week consumption of the peanuts was followed by rest, the second week each of the participants had to cycle for 60 minutes at 70% of their maximal output directly after the consumption of the sugar solution and the peanuts. The endurance exercise significantly increased intestinal permeability (lactulose/rhamnose ratio) as well as the uptake of Ara h 6. The lactulose/rhamnose ratio and the levels of Ara h6 were strongly correlated. From this it was hypothesized that endurance exercise after food consumption leads to increased paracellular intestinal uptake of food proteins. However, increased permeability due to increased transcytosis or decreased cell integrity could also add to the observed increase in the protein uptake.

Chapter 6 shows findings on the newly developed *in vitro* model for intestinal permeability. This chapter describes the development of an *in vitro* cell model mimicking to a higher extent the *in vivo* situation with regard to metabolic phenotype. The Caco-2 monolayers grown in galactose medium instead of glucose medium showed a more oxidative phenotype dependent on mitochondrial ATP production. Obstruction of this mitochondrial energy production resulted in decreased cellular ATP levels coinciding with increased monolayer permeability. Gene expression analysis of tight junction proteins was inconclusive, but pointed towards a defence mechanism of the Caco-2 cells during energy stress. It was concluded that mitochondrial functioning may be essential for maintaining a gut barrier function with high integrity.

In chapter 7 the main findings of this thesis are discussed. Furthermore, several methodological considerations are made for future design of *in vivo* studies on intestinal permeability including an exercise challenge and *in vitro* studies on intestinal permeability with Caco-2 cells. Next, future implications of my research is placed in the context of the broader aim of the IPOP Customized Nutrition research program with regard to the health effects of novel proteins. Lastly, some general conclusions of my thesis research are drawn.

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About the author

Curriculum Vitae

Lonneke Maria Janssen Duijghuijsen was born on September 17 1986 in Hoorn, the Netherlands. In 2005, she received her secondary school diploma from Oscar Romero in Hoorn.

In 2006, she started the BSc program 'Nutrition and Health', with a minor in 'Immunological Aspects of Nutrition' at Wageningen University and in 2010 she received her cum laude bachelor degree. She proceeded with the MSc program 'Nutrition and Health', specializing in nutritional physiology. She conducted a first MSc thesis in the Human and Animal Physiology group on the effects of quercetin and ambient temperature on fatty acid catabolism, followed by a second MSc thesis in Stockholm, Sweden at the Karolinska Institute on maternal nutrition and onset of allergy in the offspring, for which she received a scholarship from the Swedish 'Freemurare Barnhuset Foundation' of which the certificate was handed over by H.M. Queen Silvia of Sweden (http://resource.wageningenur.nl/nl/show/Koninklijk-eerbetoon-voor-Wageningse-studente.htm). She concluded her studies with an internship at FrieslandCampina, developing an assay for the detection of sIgA in an intestinal epithelial cell model. In 2012, she received her cum laude master degree.

In September 2012, she started her PhD project within the IPOP Customized Nutrition programme of Wageningen University and Research, working within the department of Consumer Science and Health at Wageningen Food and Biobased Research, the division of Human Nutrition, and the department of Human and Animal Physiology at Wageningen University. The project on intestinal permeability to proteins included several human and *in vitro* studies, under the supervision of Prof. Harry Wichers, Prof. Jaap Keijer, and Dr Klaske van Norren, and is presented in this dissertation.

During her PhD project, she joined the educational programme of the Graduate School VLAG. She attended several (inter)national conferences and courses and was involved in teaching activities. She supervised several students during their MSc thesis.

Currently, she is working at Wageningen University and Research within the department of Human and Animal Physiology on education and innovation in education.

List of publications

Peer reviewed publications

JanssenDuijghuijsen, L. M., van Norren, K., Grefte, S., Koppelman, S. J., Lenaerts, K., Keijer, J., Witkamp, R. F. & Wichers H. J. (2017) Endurance exercise increases intestinal uptake of the peanut allergen Ara h 6 after peanut consumption in humans. *Nutrients*, 9(1): 84. DOI:10.3390/nu9010084

JanssenDuijghuijsen, L. M., Wichers, H. J., van Norren, K., Keijer, J., Baumert, J. L., de Jong, G. A. H., Witkamp, R. F. & Koppelman, S. J. (2017) Detection of peanut allergen in human blood after consumption of peanuts is skewed by endogenous immunoglobulins. *Journal of Immunological Methods*, 440: 52-57. DOI:10.1016/j.jim.2016.11.002

JanssenDuijghuijsen, L. M., Keijer, J., Mensink, M., Lenaerts, K., Ridder, L., Nierkens, S., Kartaram, S. W., Verschuren, M. C. M., Pieters, R. H. H., Bas, R., Witkamp, R. F., Wichers, H. J. & van Norren, K (2017) Adaptation of exercise-induced stress in well-trained healthy young men. *Experimental Physiology*, 102: 86-99. DOI:10.1113/EP086025

JanssenDuijghuijsen, L. M., Mensink, M., Lenaerts, K., Fiedorowicz, E., van Dartel, D. A. M., Mes, J.J., Luiking, Y. C., Keijer, J., R., Wichers, H. J., Witkamp, R. F. & van Norren, K (2016) The effect of endurance exercise on intestinal integrity in well-trained healthy men. Physiological Reports, 4 (20) DOI:10.14814/phy2.12994

Egger, L., Ménard, O., Delgado-Andrade, C., … **JanssenDuijghuijsen, L.,** … Vegarud, G. E., Vergères, G. & Portmann, R. (2015) The harmonized INFOGEST in vitro digestion method: From knowledge to action. *Food Research International*, 88: 217-225

Expected publications

Manuscript in preparation

JanssenDuijghuijsen , L. M., Grefte, S., van Norren, K., de Boer, V. C. J., van Dartel, D. A. M., Romijnders-van der Stelt, I., Bekkenkamp-Grovenstein, M., Witkamp, R. F., Wichers, H. J. & Keijer, J. (Advanced stage of manuscript preparation) Intestinal epithelial integrity is dependent on mitochondrial ATP production: An in *vitro* model with an oxidative metabolic phenotype.

Abstracts and presentations

JanssenDuijghuijsen, L. M., Koppelman, S., Keijer, J., Mensink, M., Mes, J., Luiking, Y., Wichers, H., Witkamp, R. & van Norren, K. The effect of exercise on intestinal permeability towards small molecules and protein
Oral presentation Protein for Life conference, October 2016, Ede, the Netherlands.

JanssenDuijghuijsen, L. M., Grefte, S., Wichers, H., van Norren, K. & Keijer, J. Mitochondrial ATP production and intestinal epithelial permeability—An *in vitro* model.
Oral presentation Protein for Life conference, October 2016, Ede, the Netherlands.

JanssenDuijghuijsen, L. M., Mitochondrial ATP production and intestinal epithelial permeability—An in vitro model
Biochimica et Biophysica Acta (BBA) – Bioenergetics supplement EBEC 2016.

JanssenDuijghuijsen, L. M., Mensink, M., Witkamp, R., Keijer, J., Wichers, H., Luiking, Y. & van Norren, K. Intensive exercise hampers the postprandial citrulline bioavailability following casein bolus feeding
Poster presentation After ESPEN symposium, September 2014, Utrecht, the Netherlands.

JanssenDuijghuijsen, L. M., Mensink, M., Witkamp, R., Keijer, J., Wichers, H., Luiking, Y. & van Norren, K. Intensive exercise hampers the postprandial citrulline bioavailability following casein bolus feeding (2014).
Oral presentation 36th ESPEN congress, September 2014, Geneva, Switzerland. Clinical Nutrition supplement DOI:10.1016/S0261-5614(14)50026-X.

JanssenDuijghuijsen, L. M., Mensink, M., Witkamp, R., Keijer, J., Wichers, H., Luiking, Y. & van Norren, K. Exercise-induced intestinal permeability and dietary protein and peptide levels in blood of healthy men (2014)
Oral presentation 3rd International Conference on Food Digestion, March 2014, Wageningen, the Netherlands.

Overview of completed training activities

Wageningen University Graduate School VLAG
Food Technology Agrobiotechnology Nutrition and Health Science

Discipline-specific activities

Protein for Life conference	(Ede, NL, 2016, oral and poster presentation)
NWO Dutch Nutritional Science Days	(Deurne, Heeze, NL, 2014-2016, oral presentations)
University of Warmia and Mazury- Laboratory training	(Olsztyn, PL, 2015)
3rd After ESPEN symposium	(Utrecht, NL, 2014, poster presentation)
36th ESPEN congress	(Geneva, CH, 2014, oral presentation)
3rd International Conference on Food Digestion	(Wageningen, NL, 2014, oral presentation)
3rd Nutrition Winterschool	(Ylläs, FI, 2014, oral presentation)
Rikilt- Training Laboratory Techniques (LC-MS/MS)	(Wageningen, NL, 2013/2014)
1st INFOGEST Training School on Food Digestion	(Gdansk, PL, 2013)
2nd International Conference on Food Digestion	(Madrid, ES, 2013)
4th Advanced Food Analysis VLAG	(Wageningen, NL, 2013)
3rd INFOGEST MC&WG Meeting	(Leatherhead, UK, 2012)

General courses

GCP-IHC course	(WUR-HNE 2016)
Career orientation	(WGS 2016)
Reviewing a scientific paper	(WGS 2015)
Statistics course by Eric Boer	(WUR-FBR 2014)
Techniques for writing and Presenting a Scientific Paper	(WGS 2014)
PhD Workshop Carousel	(WGS 2014/2015)
Project and Time Management	(WGS 2014)
Did. Course: Teaching and supervising Thesis students	(ESD 2013)
PhD Competence Assessment	(WGS 2013)
PhD Week	(VLAG, Baarlo 2012)

Optional activities

FQHE/HAP/Nutrition and Pharma scientific meetings	(2012-2016)
HAP/ Nutrition and pharmacology PhD meetings	(2012-2016)
PhD Study Tour Human Nutrition	(USA 2015, oral and poster presentation)
Preparation of Research Proposal	(2012)