CLINICAL CHARACTERISTICS OF COW’S MILK ALLERGY WITH GASTROINTESTINAL SYMPTOMS

Laura Merras-Salmio

ACADEMIC DISSERTATION

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Supervisors

Docent Kaija-Leena Kolho
Children’s Hospital, Helsinki University Central Hospital
University of Helsinki
Helsinki, Finland

Professor Mika Mäkelä
Pediatric Allergy Unit, Helsinki University Central Hospital
University of Helsinki
Helsinki, Finland

Reviewers

Docent Taina Arvola
Department of Pediatrics
Kanta-Häme Central Hospital and Tampere University Hospital Allergy Center
Tampere, Finland

Docent Sami Remes
Department of Pediatrics,
Kuopio University Hospital
Kuopio, Finland

Opponent

Johanna C. Escher, MD, PhD
Department of Pediatric Gastroenterology
Erasmus Medical Center, Sophia Children’s Hospital
University of Rotterdam
The Netherlands

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ORIGINAL PUBLICATIONS
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This thesis is based on the following publications:


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Tavoite: Selvittää kaksoissokkoaltistusta hyväksikäyttäen suolisto-oireiseen lehmänmaitoallergiaan liittyviä oireita, sekä tutkia uusia suoliston tulehdusta mitaavia merkkiaineita joita voitaisiin hyödyntää taudin diagnoostiikassa. Lisäksi selvittämme vanhempien ja oireilevien lasten vuorovaikutusta ja vanhempien käsitelyksiä lapsen oireista ja niiden vaikutuksesta vanhemmuuteen.


Vuorovaikutusprofiilin poikkeavuus erityisesti altistusnegatiivisten lasten ja äitien välillä on samankaltainen kuin imeväisiän syömishäiriöissä. Pikkulapsen syömishäiriöön liittyviä oireita ovat mm. syömiseen liittyvä oksentelu ja ruuasta kieltäytyminen, jotka molemmat ovat yleisiä oireita epäiltäessä myös lehmänmaitoallergiaa. Vanhemmat kokivat usein oireilevan lapsen olevan myös temperamentiltaan vaativa ja vaikea. Löydösten valossa voidaan perustellusti epäillä äiti-lapsi-vuorovaikutuksen häiriöillä olevan merkitystä lapsen oirekuvalle, jopa siten että maidosta kieltäytyminen ja syömishetkeen ajallisesti liittyvä oksentaminen ovatkin oireita vuorovaikutuksellisesta syömishäiriöstä.
ABSTRACT

Background: Cow’s milk protein allergy (CMPA) is often suspected in infants and young children with non-specific gastrointestinal symptoms. The pathomechanisms behind the different presentations of gastrointestinal manifesting CMPA (GI-CMPA) remain unproven. Diagnosing GI-CMPA is difficult, with the gold standard of diagnosis being the double-blind, placebo-controlled food challenge (DBPCFC). However, the protocols reported in the literature are often not feasible in clinical practice. The occurrence of CMPA in Finland is 2%, and GI symptoms are reported in 20–25%.

The aim of this study is to provide a detailed clinical picture of GI-CMPA and to examine various clinical, and until recently, mainly research-based laboratory tests in order to investigate new diagnostic approaches to GI-CMPA. Little is known about the psychosocial maladjustments in patients and families suffering from GI-CMPA. Therefore, this study aims to characterise mother–child interactions and other associated psychological factors in patients suspected of GI-CMPA.

Results: This study prospectively recruited 57 patients with GI symptoms suspected of CMPA to undergo a DBPCFC for cow’s milk. The proportion of positive DBPCFCs was only 32% (18/57), and the only symptom associated with the diagnosis of CMPA was loose stools. Vomiting or regurgitation occurred in similar frequencies among the CMPA-negative and CMPA-positive patients. Excessive crying/fussing was the most common symptom among the CMPA-negative patients, reported frequently during the placebo challenges. None of the patients suspected of GI-CMPA had detectable CMP-specific IgE. Faecal calprotectin was slightly higher among the challenge-positive patients both while on a cow’s milk-free diet and after CMP provocation. The CM protein-specific blood IgG, IgG4 and IgA levels were found to be significantly low among both CMPA-positive and CMPA-negative patients while on a CMP-free diet compared to those of the control patients (without allergic or atopic diseases) who consumed cow’s milk normally. Feeding-related symptoms and problems occurred frequently.

Regarding the mother-child interaction substudy, the patients (n=24) frequently demonstrated problematic mother–child emotional interactions, especially the CMPA-negative patients (n=17). This interaction pattern was less severe but identical to the pattern found in infants with diagnosed feeding disorders. Based on the questionnaires on child behavioural characteristics, children with GI symptoms suspected of CMPA were often perceived by their mothers as being demanding and difficult, regardless of the CMPA diagnosis. Taken together, these findings suggest that the dyadic psychological attributes may augment the infant’s symptoms or the
reporting of them and may even cause feeding-related problems resulting in food refusal and feeding-related symptoms leading to suspicion of CMPA.

**Conclusions:** Gastrointestinal symptoms suspected of cow’s milk allergy are seldom confirmed when using the DBPCFC. Placebo symptoms occur frequently, the most typical being excessive crying and regurgitation. There may be CMP independent low-grade gut mucosal inflammation present in patients with positive provocation tests. Cow’s milk free diet in study infants lead to low levels of CMP specific antibodies raising concern over the safety of prolonged exclusion diets. Mother-child interaction may be problematic in patients with unconfirmed GI-CMPA, and the mothers often perceive the young children with suspicion of GI-CMPA as difficult and demanding. This psychological profile should be taken into consideration when managing patients with suspicion of GI-CMPA.
## ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AAF</td>
<td>Amino Acid Formula</td>
</tr>
<tr>
<td>CM</td>
<td>Cow’s milk</td>
</tr>
<tr>
<td>CMP</td>
<td>Cow’s milk protein</td>
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<tr>
<td>CMPA</td>
<td>Cow’s milk protein allergy</td>
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<tr>
<td>DBPCFC</td>
<td>Double-blind, placebo-controlled food challenge</td>
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<tr>
<td>EA</td>
<td>Emotional availability</td>
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<td>EAS</td>
<td>Emotional Availability Scales</td>
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<tr>
<td>EGID</td>
<td>Eosinophilic gastrointestinal disease</td>
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<td>EHF</td>
<td>Extensively hydrolyzed formula</td>
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<tr>
<td>FD</td>
<td>Feeding disorder</td>
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<td>FPIES</td>
<td>Food protein-induced enterocolitis syndrome</td>
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<tr>
<td>GER</td>
<td>Gastroesophageal reflux</td>
</tr>
<tr>
<td>GERD</td>
<td>Gastroesophageal reflux disease</td>
</tr>
<tr>
<td>GI</td>
<td>Gastrointestinal</td>
</tr>
<tr>
<td>GI-CMPA</td>
<td>Gastrointestinally manifesting cow’s milk protein allergy</td>
</tr>
<tr>
<td>IBD</td>
<td>Inflammatory Bowel Disease</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
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<td>IgA</td>
<td>Immunoglobulin A</td>
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<td>IgE</td>
<td>Immunoglobulin E</td>
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<td>IgG</td>
<td>Immunoglobulin G</td>
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<tr>
<td>ITQ</td>
<td>Infant temperament questionnaire</td>
</tr>
<tr>
<td>OFC</td>
<td>Oral food challenge</td>
</tr>
<tr>
<td>PSI</td>
<td>Parenting stress index</td>
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<tr>
<td>SCFA</td>
<td>Short-chain fatty acid</td>
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<td>SPT</td>
<td>Skin prick testing</td>
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1 Introduction

Cow’s milk (CM) is the most commonly used substitute milk for human breast milk in the Northern Hemisphere. As such, its protein contents are too high for human infants. Cow’s milk protein (CMP), however, is of good quality, and when adequately prepared to resemble human breast milk protein and fat contents, cow’s milk-based infant formulas are generally well tolerated by infants and promote good health and growth in infants for whom breast-feeding is not feasible. Less than 2% of children will unfortunately develop an allergy (or intolerance) to cow’s milk protein. Reactions related to having a cow’s milk protein allergy (CMPA) are typically manifested in the skin as eczema and/or urticaria, or in extreme cases, as anaphylaxis, a multi-system, life-threatening allergic reaction. Such reactions can be mediated by the immunoglobulin E (IgE) class of antibodies.

Some children present with gastrointestinal symptoms, which occur, depending on the study setting, in 20–50% of children with physician-diagnosed CMPA. Typical gastrointestinal symptoms suspected by parents and physicians to be caused by cow’s milk include colicky crying, vomiting and gastroesophageal reflux, diarrhoea and constipation. However, such symptoms occur in more than 50% of healthy infants as well. There are currently no laboratory tests available that can accurately and specifically diagnose GI-CMPA. Skin prick testing and allergen-specific IgE measurements only concur with the IgE-mediated allergy. The gold standard in diagnosing any food allergy is the double-blind, placebo-controlled food challenge (DBPCFC). As this procedure requires a careful methodology and takes up to three weeks to perform, it is unfortunately seldom used in practice and clinicians rely on the open food challenges instead, even though open challenges are unreliable for diagnosing non-IgE CMPA.

Little is known about the psychology associated with the non-specific, but often debilitating, gastrointestinal symptoms leading to a suspicion of GI-CMPA in infancy and early childhood. There is a recognised phenomenon of some parents not being able to reduce the allergy diet restrictions after negative food challenges, the reasons for which are poorly understood. A connection may exist between the diagnosis of GI-CMPA and the development of infant feeding-related symptoms and problems. In other related gastrointestinal disorders, researchers have found associations between maternal anxiety and the development of a disease-related feeding disorder. Therefore, it is likely that infants with gastrointestinal symptoms suggestive of CMPA are also at risk of experiencing psychosocial maladjustment.
2 Review of the literature

2.1 Overview of the infant immune system and the acquisition of tolerance to food antigens

The foetal immune system must protect the foetus from microbial infections as well as be prepared to mediate the transition to the extrauterine, antigen-rich environment [Belderbos 2009; Levy 2007]. Its functions are set to avoid the Th1 pathway (Figure 1) of the immune response (which might result in enhanced pro-inflammatory reactions), allegedly because Th1 mediated reactions may possibly result in alloimmunization processes in the foetus and preterm labour [Battersby 2013; Levy 2007; Randolph 2005]. Postnatal exposure to microbial antigens, especially lipopolysaccharides, exerts in the newborn a controlled, low-level Th1 response, which is modulated by the immunological components of human breast milk [Battersby 2013; Friedman 2005; Levy 2007]. In fact, the commonly accepted hygiene hypothesis states that frequent exposure to microbial antigens during early childhood (including intrauterine life) may accelerate the transition from the Th2 preference to Th1-mediated immune responses, thus reducing the risk of developing atopic diseases [Abbas 2011; Levy 2007].

In the neonate, the gut mucosa is the recipient of a remarkable microbial (and later, in infancy, also nutrition-derived) antigen load. The process of microbial colonisation is mediated by several pathways, with the end result being commensal bacterial antigen acceptance and tolerance [Battersby 2013; Miron 2012]. In preterm infants, such mediation is deficient, with the subsequent increased risk of uncontrolled, Th1-mediated inflammatory reactions predisposing the infant to necrotising enterocolitis (NEC), a life-threatening newborn gut mucosal disease [Battersby 2013; Weitkamp 2013]. In term infants, there is extensive crosstalk between the different immune system cells in the intestine as maturation and commensal bacterial colonisation proceeds, with the aim being to produce controlled immune responses. This crosstalk is affected via cytokines, such as TGF-β, IL-10 (anti-inflammatory and tolerogenic effects), IL-17 and IFN-γ (pro-inflammatory) [Abbas 2011; Battersby 2013; Randolph 2005]. A subset of T cells called γδ cells, which usually do not express surface molecules inherent to other specific T cell
populations, typically resides in the intestinal epithelium. These cells form a connection with the innate and adaptive immune responses by employing their antigen-presenting skills and by directly secreting proinflammatory cytokines and immunomodulatory functions upon coming into contact with peptide antigens [Su 2013]. An increase in γδ-cells was noted in the epithelium of patients (both IgE and non-IgE) with food allergies [Westerholm-Ormio 2010].

**Figure 1** The differentiation of Th1 and Th2 cells and their main effector cytokines. Th1 activation is driven by IL-12 produced by antigen-presenting cells, and Th2 activation by IL-4 produced by naive T cells. The recently identified Th-subsets Th9, Th17 and T22 emphasise the multitude of active players, both proinflammatory and tolerogenic, involved in the immune processes.
The effects of food-derived antigens on the infant’s gut mucosal system are not uniform, resulting in conflicting outcomes in studies addressing the role of pregnant and lactating mothers’ diets and the overall protective impact of breastfeeding [Friedman 2005]. Non-atopic mothers’ use of high amounts of CM decreased CMPA diagnoses among their offspring [Tuokkola 2011], whereas maternal peanut consumption correlated with increased rates of IgE sensitisation to peanuts among the offspring [Sicherer 2010]. Immunological (genetic) factors in newborn infants, the dose and timing of food allergen exposure, as well as environmental factors (including parental stress [Wright 2005]), are therefore all involved in the process of directing the immune responses to food antigens [Belderbos 2009].

Furthermore, the gut mucosal barrier functions are vulnerable during infancy, with increased permeability during the newborn period. The intestinal barrier includes the mucus secreted from the goblet cells, several antimicrobial peptides (e.g. lysozymes, defensins, lactoferrin, calprotectin) secreted from Paneth cells, enterocytes and mucosal granulocytes, as well as the prevention of bacterial translocation by the tight junctions between enterocytes and the surveillance functions of all epithelial antigen-recognising cells [Abbas 2011; Battersby 2013].

Antibodies (immunoglobulins) are produced in the B cells, but this humoral response is rather ineffective in newborns [Abbas 2011; Bonilla 2010]. As maternal IgG actively crosses the placenta, the foetus and the newborn have an abundance of maternal-origin IgG to help fight extracellular pathogens. B cells produce immunoglobulins according to the cytokine environment they are in. IgE production is promoted especially by IL-4 (a cytokine typically secreted by activated Th2 cells), and counteracted by IFN-γ (a typical Th1 cell-produced cytokine). IgE production is thus part of a natural response to new antigens, especially in early childhood [Abbas 2011; Randolph 2005]. The IgE antibodies can bind to IgE-receptors (Fcεr) on the surface of mast cells, eosinophils and basophils, and when encountering a specific antigen, they cause these cells to secrete potent proinflammatory substances, such as histamine, leukotrienes, eosinophilic cations and a wide array of cytokines [Gould 2008; Yan 2009].

Tolerance to microbial and food-derived antigens develops gradually as the infant’s immune system matures. In the foetus and newborn, the thymus is involved in processing the lymphocytes so that the T lymphocytes primed against self-antigens are discarded [Bonilla 2010; Randolph 2005]. For food protein-induced enterocolitis syndrome (FPIES) patients who have gained a tolerance to such antigens, T-regulatory cells have been shown to play a major role in the suppression of the proinflammatory cells via direct cell-to-cell contact (with both effector T cells and antigen presenting cells) and by enhancing the production of TGF-β and IL10 [Caubet 2011]. CD4+CD25+ T-regulatory cells are activated by the transcription factor FOXP3. A genetic disease lacking FOXP3 (IPEX syndrome) results in severe
IgE-mediated food allergies and worsening atopic eczema [Abbas 2011]. The role of FOXP3 mediation seems to help maintain tolerance and control the intensity of the allergic response rather than the sensitisation process itself, the details of which are still largely unknown [Saurer 2009].

Humoral responses associated with the gaining of tolerance include an increase in serum levels of specific IgG4 and IgA [Wisniewski 2013]. With IgE-mediated allergies, the proportion of IgE to IgG4 diminishes as tolerance develops [Savilahti 2010a; Wang 2011]. IgG4 aids in promoting tolerance presumably by 1) binding weakly to the immunoglobulin receptor Fc and thus preventing the antigen from binding to the receptor, and 2) by inhibiting the complement activation associated with an IgE-mediated response [Wisniewski 2013]. Different (as well as naturally modified, e.g. cooked) antigen peptides from the same food (or another allergen) trigger non-identical responses in the host, with some peptides even having tolerogenic properties [Kim 2011; Wang 2011; Wisniewski 2013]. Research on the tolerance mechanisms is ongoing and further information is still needed to fully understand this process.
2.2 Immunological aspects of cow's milk protein allergy

2.2.1 The immune response to cow’s milk proteins

Cow’s milk proteins are divided into two parts: whey proteins (approximately 20%) and caseins (80%). Of these proteins, beeta-lactoglobulins (a whey protein) and alpha-caseins in particular are implicated in the allergic immunopathology. All CMPs are capable of mounting an immune response, though [Wal 2004]. With IgE-mediated CMPA, alpha-caseins may be the most allergogenic of CMPs [Schulmeister 2009]. Interestingly, mainly bovine lactoglobulins have been found in human breast milk after CM has been consumed by the lactating mothers [Axelsson 1986; Coscia 2012], but this finding is not unequivocal [Restani 2000]. The mechanisms of immune responses to specific CMPs in non-IgE or gastrointestinally manifested CMPA are yet unproven, and they most likely represent more than one pathway [Caubet 2011].

2.2.2 IgE-mediated hypersensitivity reactions

In health, there is a delicate balance between the necessity of fighting pathogens and recognising non-pathogenic (commensal) microbes and harmless food antigens. The skin and gut mucosal barriers have a major role in this process. Intestinal epithelial cells and dendritic cells that act as antigen-presenting cells on the gut mucosa are in fact ‘built’ to favour tolerance [Berin 2013]. The epithelial cells lack the co-stimulatory signal needed to amplify the immune response elicited by the binding of a luminal antigen to their major histocompatibility complex receptor. The dendritic cells in turn mostly express IL-10 and IL-4, which also favours tolerance of mucosal commensal bacteria by inhibiting TH1-mediated immunity [Abbas 2011; Sicherer 2009]. There is strong evidence suggesting an imbalance between Th1- and Th2-mediated responses in infants with IgE-mediated allergies [Schade 2000]. The pathophysiologic process in IgE-mediated allergy reactions is well documented. An activated Th2 cell produces even more IL-4, which also promotes IgE production in the B cells. Specific IgE then coats the mast cells, and upon subsequent exposure to a particular antigen, causes the mast cell to become activated. Activated mast cells secrete bioactive amines (such as histamine), prostaglandins and leucotrienes, and cytokines (IL-4, IL-5, IL-13). Eosinophils are then recruited, especially by IL-5 and IL-13. The late-phase reaction also includes the recruitment of macrophages, which are activated by the Th2 response and produce the extracellular matrix proteins.
involved in tissue repair, and which are actually inhibited from engaging in a Th1-mediated response to microbicidal activities [Sicherer 2009]. This type-I hypersensitivity reaction begins with the first exposure to an allergen, and upon re-exposure, a rapid cascade of immune responses elicits the typical symptoms of a mucocutaneous allergy. Why in some individuals this cascade results in an uncontrolled severe anaphylaxis reaction is still not completely understood. The levels of specific IgE in serum reflect sensitization and not clinical reactivity to a specific food. Clinical FA probably occurs in IgE-mediated pathways only if the concomitant tolerogenic mechanisms break down [Wang 2011].

2.2.3 Non-IgE-mediated reactions

In early childhood, severe gastrointestinal reactions to food antigens are known to occur also in the absence of IgE production. In FPIES patients, researchers have found evidence of pronounced T cell-mediated inflammatory reactions: there are increased intraepithelial lymphocyte counts in the small intestinal mucosa and evidence of macrophage activation. Mucosal lymphocytes in FPIES exhibit increased TNF-a (and synergist IFN-γ) production combined with a subdued TGF-β response, causing increased gut permeability, which allows for further activation of antigen-specific T cells and subsequent proinflammatory cytokine production, resulting in clinically severe symptoms [Caubet 2011]. Related evidence has been found also in a small series of delayed-type CMPA with less severe symptoms and increased IFN-γ staining in duodenal lymphocytes [Veres 2003]. Since IFN-γ is produced mainly by the Th1 cells, it is possible that the cellular response in the delayed-type CMPA manifesting in the intestine represents a dip towards the other end of the Th1/Th2 dysbalance. Th1 –produced cytokines (IFN-γ, TNF-a) increase intestinal permeability, which aggravates the inflammatory reaction [Berin 2013]. A simultaneous decrease in TGF-β fails to counterbalance this process. The role of regulatory T cells is again substantial, and their numbers increase as tolerance develops, with TGF-β and IL-10 increasingly being produced. Macrophage activation via the Th1 pathway results in full activation with ensuing cytokine and chemoattractant production, while polymorphonuclear cells (neutrophils) and eosinophils are also recruited. Activated neutrophils then degranulate and secrete antimicrobial agents such as calprotectin and lactoferrin [Abbas 2011].

A food allergy associated with primary eosinophilic gastrointestinal disorder (EGID) likely represents a mixed IgE/non-IgE etiology. The Th2-derived cytokine IL-5 plays a significant role in the activation and maturation of eosinophils, and the mostly intestinal epithelial cell-derived chemotaxins (eotaxins) then recruit eosinophils to the gut mucosa. The Th2 pathway includes IgE-mediated processes, as befitting the significant concurrence of sensitisation, but in the intestine it seems that eosinophilic inflammation is fairly often triggered by direct cell-to-cell contacts [Yan 2009].
2.2.4 Immunoglobulins IgG(total), IgG1, IgG4 and IgA in CMPA

The activated B cell migrates from the gut mucosa to the lymph node germinal center, where the immunoglobulin class switch takes place. In an IL-4-dominated environment, IgE is produced. The presence of IFN-γ promotes IgG1 and IgG3, whereas IL10 promotes IgG4 and TGF-β in turn promotes an IgA class switch [Abbas 2011]. IgM is produced through the innate immune system (complement activation). IgA is the principal antibody secreted through mucosal epithelia, neutralising luminal antigens. Increased faecal cow’s milk-specific IgA was indeed shown to correlate with reduced sensitisation rates among children prone to atopy [Kukkonen 2010]. The role of IgG (and the subtypes IgG1 or IgG3) in allergic pathophysiology is ambivalent. Increased levels of circulating cow’s milk-specific IgG and IgG1 were found both in symptomatic patients and non-symptomatic controls [Hochwallner 2011]. There are no data to support the use of specific IgG measurements in the diagnosis of food allergies [Boyce 2010]. It is postulated that the presence of food antigen-specific IgG is associated with increased intestinal permeability rather than with the immunopathologic process. The production of specific IgG4 is driven by IL10, which is secreted by Th2 cells as well as by, e.g. macrophages, and has a role in depressing the Th1 pathway. IgG4 may bind to the IgE antigen receptor, thereby preventing antigen binding and the subsequent triggering of the mast cell activation. In patients who have become tolerant to CMP after experiencing a previous IgE-mediated allergy, the proportion of IgG4 to IgE increases significantly [Savilahti 2010a]. IgG4 production is generally rather inefficient in infancy, with adult levels only being reached during school age [Abbas 2011]. Low levels of cow’s milk-specific IgG4 have previously been both linked [Shek 2005] and not linked [Hochwallner 2011] to non-IgE CMPA.
2.3 Gut mucosal inflammation in CMPA

2.3.1 Histology of gut mucosa in CMPA

Unfortunately, large-scale controlled studies looking into the gut histology in non-IgE-mediated CMPA are lacking. An obvious explanation is the need to sedate or provide children with general anaesthesia when obtaining biopsy samples via esophago-gastro-duodenoscopies and ileocolonoscopies. A study published in 1975 reported on 31 infants with malabsorption and diarrhoea (with concurrent vomiting in 16/31, and eczema in 6/31) diagnosed with early-onset (within the first weeks of life) CMPA. Subtotal or partial villous atrophy of the jejunum was found in 26 patients. Eosinophilic infiltrates were not common, and the authors noted that the histology they were describing was similar to various other gastrointestinal disorders experienced during childhood [Fontaine 1975]. In contrast, Berg et al. studied pre- and post-CMP challenge jejunal biopsies in eight children and did not find any discernible changes between the two [Berg 1979]. An expert review in 2000 evaluated the findings of mucosal pathology in CMPA [Savilahti 2000]. Approximately 50% of the studies on CMPA histology published at that point reported villous atrophy, with a clear decreasing trend over the years (only 15% in the more recent studies). Increased numbers of intraepithelial lymphocytes were reported for CMPA (both IgE and non-IgE) patients, especially cytotoxic T cells and some eosinophila. Epithelial cells (especially crypt cells) showed high mitotic activity, with their immaturity associating with low disaccharidase activity and signs of secondary lactase malabsorption. A more recent study noted the prominence of intraepithelial γδ T cells [Kokkonen 2001; Westerholm-Ornio 2010]. Infants with hematemesis (n=8) who were also suspected of having CMPA had an upper GI endoscopy performed, with histological findings of a significant erosive duodenal bulb and antral lymphoid hyperplasia. Only 1/8 had villous atrophy in the duodenum, and 3/8 showed signs of reflux esophagitis in the esophageal biopsies (none had remarkable esophageal eosinophilia) [Al-Hussaini 2012].

It has been suggested that intestinal lymphoid hyperplasia is related to food allergies in school-aged children [Kokkonen 2002]. In children (age 1–15 years) with recurrent abdominal pain, a duodenal endoscopy was more likely to result in macroscopic lesions among children with food allergies (a food allergy was diagnosed in 33% of the patients) than among the non-allergic children, with the respective figures being 39% and 11%. However, eosinophilic infiltrates were similarly frequent in both groups, regardless of the anatomical region [Kokkonen 2001]. In another study of 35 constipation-prone patients aged 3–15 years, 34% of patients exhibited a positive elimination-challenge response to CMPA. Intestinal lymphonodular
hyperplasia of the colon was present in 46% of patients, while there was an increased ratio of \(\gamma\delta\) T cells to CD3+ T cells in the ileum. There was also increased eosinophilia in the terminal ileum. Unfortunately, the study did not distinguish between patients with food allergies and those without food allergies. In control patients without constipation or another specific gastrointestinal diagnosis, lymphonodular hyperplasia (LNH) was present in the colon in only 1/15 and terminal ileal eosinophilia in 7/15 patients. An atopic disease was present in 34% of the study patients compared to 20% of the controls [Turunen 2004].

An increasing number of studies are now focusing on the histology of eosinophilic gastrointestinal disorders (EGID). The diagnosis for EGID is traditionally based on the histological finding [Yan 2009]. The lack of age-related normal reference values for gut mucosal eosinophils should be noted; furthermore, the extent to which eosinophilic disorders correlate with actual food allergies has not yet been proven. However, there is increasing consensus among experts on the diagnostic criteria for eosinophilic esophagitis, including both increased eosinophil counts in the esophageal mucosal (> 15/HPF) and a suitable clinical presentation, while emphasising the fact that eosinophilic esophagitis is not a solely histopathological diagnosis [Liacouras 2011].

2.3.2 The role of intestinal bacterial flora in suspected CMPA

There is increasing evidence to support the fact that gut commensal microbiota plays a major role in the pathogenesis of atopic/allergic disease. Faecal samples taken at 3 weeks and 3 months of age were different for sensitised SPT-positive one-year-old children than for non-sensitised children; they had more \textit{Clostridia} and fewer \textit{Bifidobacteria} [Kalliomaki 2001]. The direction of effects in vivo is still being debated. In a study of mice, knockout mice with genetically allergy-prone eggs showed a special gut microbiota signature early in life. This signature was further enhanced when the mice were sensitised, but it changed again to a distinct tolerance-associated signature after suppression of the allergic response. Most interestingly, transferral of the allergy-prone gut microbiota to germ-free, wild-type mice promoted specific IgE responses in the recipients [Noval Rivas 2013]. The reverse was also true: allergic mice infused with healthy human infant microbiota exhibited tolerogenic responses and a reduced number of symptoms during re-challenges [Rodriguez 2012].

In atopic/allergic children, faecal microbial analyses at an early age have showed changes in the composition of gut microbiota, which differs from the analyses of children without atopy at follow-up [Nakayama 2011; Sjogren 2009]. Some proteobacteria and clostridia species were more abundant later in non-allergic
children at one month of age, while early colonisation with bacteroides species associated with allergies [Nakayama 2011]. In all above-mentioned studies, colonisation with bifidobacteria and proteobacteria seems to be beneficial. The proteobacterial lipopolysaccharide has a strong immunological effect, which may be important for the developing immune system, as suggested by the hygiene hypothesis [Nakayama 2011].

In clinical studies, tolerance to CMP in non-IgE CMPA is shown to develop faster if the formula contains the probiotic lactobacillus strain LGG [Berni Canani 2013a]. Nevertheless, the role of probiotics in the prevention and treatment of food allergies has been disappointing despite exhaustive data proving a positive effect, since the clinical value remains limited [Canani 2013]. An important factor is the remarkable difference in the clinical effect between the different probiotic strains. Another point is the diversity of normal gut microbiota, with more than 1000 microbial subspecies having already been identified.

Since present day suspicions of CMPA (in the absence of cutaneous manifestations) are often based on colic, the role of microbiota in a colicky infant is worth discussing. Colicky infants also often exhibit aberrant faecal microbiota, with similarities to patterns seen in food allergies. The lactobacilli and bifidobacteria species in particular are less abundant, with the overall microbial diversity also being lower in the faecal samples of infants with colicky crying [De Weerth 2013; Partty 2012]. Possible mediating factors include subtle inflammation in the gut mucosa, as suggested by somewhat increased faecal calprotectin values in colicky infants [Rhoads 2009], and the role of short-chain fatty acids (SCFA) in the metabolism of intestinal microbiota producing SCFAs (butyrate, acetate and propionate). SCFA function as energy substrates for the colonocytes, they modulate colonic pH, they regulate colonic cell proliferation and differentiation, and they contribute to hepatic gluconeogenesis and cholesterol synthesis [Wong 2007]. SCFA (butyrate) may also help modify pain in the colon, the direction of which may depend on the amount of the substrate present [Kannampalli 2011].

### 2.3.3 Stool markers of inflammation

Calprotectin is the major neutrophil cytoplasmic protein, constituting approximately 60% of granule proteins. To a lesser extent, it is also excreted by macrophages and monocytes. Calprotectin is a calcium-binding protein with multiple, though yet not precisely understood, roles in the immune defence system, involving apoptosis regulation, microbicidal activity and various immunomodulatory functions [Steinbakk 1990]. Calcium binding makes this molecule resistant to heat and
proteolysis, thereby ensuring its stability in stool samples at room temperature for up to seven days. Levels of faecal calprotectin increase whenever there is increased neutrophil accumulation in the gut mucosa, making it a sensitive surrogate marker for intestinal neutrophilic inflammation and a helpful tool in the diagnosis and follow-up of Inflammatory Bowel Disease (IBD). Calprotectin levels also increase in acute microbial intestinal infections, with colorectal cancer and with bloody stools [Gisbert 2009; Henderson 2013]. Faecal calprotectin levels are often high during infancy, without any signs of ongoing disease. Exclusively breastfed infants have significantly higher levels of faecal calprotectin at 1–3 months of age compared to formula-fed infants [Savino 2010]. In a small study utilising only the open food challenge to diagnose CMPA, calprotectin levels did not differ between CMPA patients and GERD/clinical gastritis patients [Kalach 2013]. In allergy-prone children, higher faecal calprotectin levels at six months reduced the risk of later atopic sensitisation [Kukkonen 2010].

Human $\beta$-defensins belong to a group of antimicrobial peptides, with pleiotropic functions in the mucosal innate defence system. They exhibit distinct microbicidal activity (sometimes referred to as endogenous antibiotics), but also other immunomodulatory functions affecting, e.g. wound closure, angiogenesis and the activation and recruitment of various immune system cells [Kapel 2009; Zilbauer 2010]. Human $\beta$-defensin-1 is constitutently expressed by the intestinal epithelial cells under physiological conditions. Human $\beta$-defensin 2 is an inducible molecule, which has been shown to increase in the colonic mucosa of patients with IBD, especially ulcerative colitis, but less so in Crohn's disease. In adult patients suffering from irritable bowel syndrome (IBS), $\beta$-defensin 2 levels were also found to be elevated compared to those of the symptom-free controls, but lower than those of the IBD patients, suggesting a possible microbial etiology for IBS symptoms in adults [Langhorst 2009].

Faecal eosinophil-derived proteins have also been studied with respect to CMPA. Given their connection with IgE-mediated food allergies and eosinophils, eosinophil cationic protein (ECP) and eosinophil-derived neurotoxin have been studied to find a surrogate marker for allergic inflammation in the gut. Their utility in GI-CMPA has not yet been confirmed. Saarinen et al. studied 206 infants suspected of having CMPA based on a positive CMP elimination response. One hundred two (50%) of the infants had a positive open OFC result, while only 22 of them exhibited gastrointestinal symptoms. Compared to the challenge-negative patients, the mean faecal ECP in the challenge-positive patients was higher before the CMP challenge (p=0.09), but similar (p=0.96) post challenge. A small but significant increase in ECP levels was noted in late (> 24 hrs) reactors versus immediate reactors (p=0.02) and in those with gastrointestinal symptoms (versus those without, p=0.009). However, ECP levels were generally lower in samples taken after the CMP challenge [Saarinen 2002]. Kalach et al. studied a smaller number of infants with suspicion of GI-CMPA based on medication-refractory reflux symptoms and ‘clinical gastritis’
symptoms (n=25). The open OFC was positive in 11/25 of the infants, of which 8/11 were non-IgE mediated challenges. In the challenge-positive patients (compared to the CMPA-negative patients), the pre-challenge levels of faecal eosinophil-derived neurotoxin were somewhat higher. A cow’s milk-free diet was not uniformly employed and no post-CMP provocation tests were taken. The Kalach study also reported on faecal calprotectin, TNF-a, β-defensin 2, α-1-antitrypsin and secretory IgA, none of which showed significant between group differences at any point in time [Kalach 2013].
2.4 Clinical aspects of cow's milk protein allergy

2.4.1 Symptoms associated with cow’s milk protein allergy

Cow's milk protein allergy (CMPA) presents most frequently (in more than half of the patients) in the skin with urticarial erythema and atopic, dermatitis-like eczema. This form of CMPA is typically mediated by cow's milk-specific IgE, and the suspicion can be substantiated by positive skin prick testing and significantly elevated cow’s milk-specific IgE levels in the blood (Table 1). For both the skin prick tests (SPTs) and the cow’s milk-specific IgE levels, there are distinct predictability curves. For example, the reported 95% confidence interval upper limits for a positive cow’s milk challenge were 12.8 mm for the SPT [Verstege 2005] and ≥32 kU/l for specific IgE [Sampson 1997]. The diagnosis is always made via an oral food challenge (OFC) using cow’s milk (CM). Infants and young children with IgE-mediated disease usually react immediately to the food challenge (i.e. the reaction starts within a few hours of consuming CM) and the use of open food challenges is reasonable since it is possible to objectively interpret the elucidated symptoms. Between 10 and 25% [Baehler 1996; Hill 1986] of cutaneous manifestations of cow’s milk allergy are not mediated by IgE, and in such cases the response to CM provocation generally occurs more slowly (within 24–72 hours) compared to those with IgE-mediated allergies, often necessitating the use of the double-blind, placebo-controlled food challenge (DBPCFC) to rule out confounding factors.

Gastrointestinal symptoms occur in 20–50% of CMPA patients [Hill 1986; Host 1988; Saarinen 1999; Vanto 1999]. In a prospective Finnish birth cohort of 6209 infants studied by Saarinen et al., the overall cumulative incidence of CMPA was 1.9%, of which 14% (17/118) of the infants presented with non-IgE-mediated gastrointestinal symptoms during the challenge [Saarinen 2000]. They did not use the DBPCFC. Thus, the incidence of gastrointestinally manifesting, delayed-type CMPA in Finland could be estimated to be at or below 0.27%.

The rather wide variation in the gastrointestinal symptoms reported in literature likely reflects the diagnostic challenges associated with the gastrointestinal manifestations. The most severe form of gastrointestinally manifesting CMPA (GI-CMPA) is FPIES. FPIES is characterised by repetitive vomiting (within 24 hours of ingesting cow’s milk), which is sometimes associated with pallor and even shock, followed by diarrhoea. The occurrence of FPIES was estimated at 0.34% and that of IgE-mediated CMPA at 0.27% in recent studies of Israeli infants and young children.
The age of FPIES patients is young: the median age at onset was 30 days in the Katz cohort. In fact, one of the diagnostic criteria for FPIES is the onset of symptoms before the age of 6–9 months [Katz 2011; Sicherer 2000a]. In present-day clinical work, milder forms of gastrointestinal symptoms account for the majority of GI-CMPA suspicions. Typical symptoms suspected of GI-CMPA include 1) vomiting or gastroesophageal reflux disease (GERD) symptoms, 2) colicky crying and excessive fussing, 3) constipation, 4) rectal bleeding and 5) chronic diarrhoea.

Vomiting/GERD in concurrence with CMPA has been reported in the existing literature (Table 1). In some individuals, CMP may cause either vomiting (including retching) or troublesome gastroesophageal reflux (GER). FPIES notwithstanding, the association of CMPA and vomiting/GERD is not implicit and the pathophysiology behind the symptoms remains unproven. The frequency of this association is rare: recently, CMPA was diagnosed (in a prospective follow-up study) in 1/210 infants with GER symptoms in primary care [Campanozzi 2009]. Of the 85 CMPA patients described in the largest study to date addressing the connection between GERD and CMPA, the OFC also provoked diarrhoea in 74/85 (87%) of patients [Iacono 1996]. The pH studies have been conducted with a relatively small number of patient and offer conflicting results (see Table 1). The protein contents of infant milk as such correlate with the infant GER symptoms [Aceti 2009]. Lately, the increased use of acid suppression medication in young children has introduced a new issue: the use of acid suppression increases the risk of a food allergy, both IgE-mediated and non-IgE-mediated[Trikha 2013].

Colicky crying is often suspected to be associated with ingesting cow’s milk protein. Table 2 describes studies that address this association. The strongest contradictory evidence comes from a prospective follow-up study, where the occurrence of colicky crying (9.2%) in breastfed infants and formula-fed infants (7.6%) was in fact similar (p=0.40) [Castro-Rodriguez 2001]. Colicky crying, as a sole manifestation of CMPA, is unlikely to be caused by CMPA [Clifford 2002; Lucassen 2010]. There is increasing evidence that in addition to psychological factors [Canivet 2000; Douglas 2011; Taubman 1988], gut microbiome [De Weerth 2013; Partty 2013b; Roos 2013] and functional GI disorders [Partty 2013a] may also play a role in the etiology of excessive crying during infancy.

Cow’s milk protein is widely believed to promote constipation in the human intestine, although confirmatory data are scarce. The usual culprit has been the β-casein fraction in CM. Prospective studies have not confirmed the initial Italian research on this topic, which found an association between childhood (mean patient age was 34 months) constipation and CMPA [Iacono 1998]. Most of the patients in that study had IgE-mediated CMPA and a history of atopy, while one-third had a previous suspicion of cow’s milk allergy. The pathophysiology for such an association is still being debated [Crowley 2013; Vandenplas 2012].
<table>
<thead>
<tr>
<th>Study Reference</th>
<th>Study Design</th>
<th>Participants</th>
<th>Endpoint</th>
<th>Result</th>
<th>Double-blinding</th>
<th>GERD Diagnosis</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td><a href="Palermo">Cavataio 1996</a></td>
<td><a href="Palermo">Cavataio 1996</a></td>
<td>n=96</td>
<td>Endpoint: No. of patients with CMPA, and pH monitoring in children with suspicion of GERD.</td>
<td>Result: 14/96 diagnosed with CMPA+GERD, 25 CMPA only, 33 primary GERD, and 24 with other diseases causing vomiting. A phasic pattern of pH monitoring was present in 36/39 CMPA patients and 0/57 other GERD patients.</td>
<td>Double-blinding used, 24-hour challenge, not systematically placebo-controlled.</td>
<td>GERD diagnosis based on histology (esophagitis, n=47/89) or abnormal pH-monitoring (not defined).</td>
<td></td>
</tr>
<tr>
<td><a href="Palermo">Iacono 1996</a></td>
<td><a href="Palermo">Iacono 1996</a></td>
<td>n=204</td>
<td>Endpoint: No. of patients with positive OFC in infants with GERD evaluated in a tertiary hospital.</td>
<td>Result: CMPA diagnosed in 85/204*. Total reflux time, and nr. of reflux episodes were similar in CMPA+GERD and GERD-only patients. Reflux and vomiting occurred with similar frequency in CMPA and non-CMPA.</td>
<td>Double-blinding used, 24-hour challenge, not systematically placebo-controlled.</td>
<td>GERD defined as Reflux Index &gt; 5.2% or esophagitis histology.</td>
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<tr>
<td><a href="Palermo">Ravelli 2001</a></td>
<td><a href="Palermo">Ravelli 2001</a></td>
<td>n=16</td>
<td>Endpoint: Evaluation of gastric motility after challenge with CMP.</td>
<td>Result: Gastric dysrhythmia was more frequent in CMPA (n=7) patients (71% vs 33%, p&lt;0.001).</td>
<td>DBPCFC not used.</td>
<td>GERD defined clinically, no pH studies.</td>
<td></td>
</tr>
<tr>
<td><a href="Palermo">Nielsen 2004</a></td>
<td><a href="Palermo">Nielsen 2004</a></td>
<td>n=42</td>
<td>Endpoint: No. of GERD patients with positive OFC for cow’s milk.</td>
<td>Result: 18/42 had GERD, 10/18 had positive OFC (2/10 age &lt; 1 y). Total reflux time was longer in the CMPA group, but no. of refluxes the same.</td>
<td>DBPCFC (48-hour) used if age &gt; 3 years, otherwise open OFC.</td>
<td>GERD diagnosis based on reflux index &gt;10% or esophagitis histology.</td>
<td></td>
</tr>
<tr>
<td><a href="Palermo">Borrelli 2012</a></td>
<td><a href="Palermo">Borrelli 2012</a></td>
<td>n=17</td>
<td>Endpoint: Esophageal pH and impedance monitoring in CMPA patients with suspected GERD during challenge with CMP.</td>
<td>Result: Weakly acidic reflux more frequent (16 episodes vs. 43, p&lt;0.001) during CMP. The total reflux time and the total number of reflux episodes were similar during use of AAF and CMP.</td>
<td>DBPCFC not used; all patients on AAF**.</td>
<td>GERD suspicion only; no definitive diagnosis required.</td>
<td></td>
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</table>

Table 1. Studies reporting the connection of gastroesophageal reflux disease (GERD) with cow's milk protein allergy (CMPA). AAF Amino acid formula.

*IgE positivity not reported but 48/85 dermatitis (p<0.001). Diarrhea in 74/85 at OFC (p<0.001).

** Diagnosis based on open OFC, no previous history or IgE status provided.
<table>
<thead>
<tr>
<th>Reference</th>
<th>n</th>
<th>Design</th>
<th>DBPCFC</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lothe 1989</td>
<td>24</td>
<td>One-day DBPCFC. Added CM protein powder in regular CM. Drop-outs n=3</td>
<td></td>
<td>Crying hours were reduced in CM free group, but also (less) in the placebo group.</td>
</tr>
<tr>
<td>Jakobsson 2000</td>
<td>15</td>
<td>DBPCFC not used.</td>
<td></td>
<td>Crying hours reduced in CM free group compared to baseline (no control group)*.</td>
</tr>
<tr>
<td>Lucassen 2000</td>
<td>38</td>
<td>DBPCFC not used.</td>
<td></td>
<td>Crying minutes reduced slightly in the whey hydrolysate group (p=0.05); no difference in overall responder status after one week (p=0.65).</td>
</tr>
<tr>
<td>Taubman 1988</td>
<td>19</td>
<td>DBPCFC not used.</td>
<td></td>
<td>Crying hours decreased faster in the group receiving counseling (p&lt;0.02), no effect with dietary CM restriction**.</td>
</tr>
<tr>
<td>Iacono 1991</td>
<td>70</td>
<td>DBPCFC not used.</td>
<td></td>
<td>Crying hours reduced with soy formula by more than 2 hours in 77% (p-values or confidence intervals not reported).</td>
</tr>
<tr>
<td>Hill 2005</td>
<td>104</td>
<td>DBPCFC not used.</td>
<td></td>
<td>Crying hours reduced more in the maternal low-allergen-diet group (p&lt;0.01). Nr. of patients crying &gt;360 min/48h at days 8-9 similar in both groups (p=0.402).</td>
</tr>
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</table>

**Table 2.** Studies addressing the association of colicky crying and cow’s milk protein allergy (CMPA).

* Study aim was to compare two different casein hydrolysates (no difference found).
** Casein hydrolysate formula or maternal CM free diet.
*** 16/50 of the CMPA positive had concurrent diarrhea or vomiting; and 21/50 stool blood positive.
Studies addressing infant feeding have shown that cow’s milk formulas produce harder stools compared to breast milk and hydrolysed formulas [Bongers 2007; Lloyd 1999; Mihatsch 2001; Quinlan 1995]. Cow’s milk proteins may cause gastric dysrhythmia, including slow gastric emptying [Ravelli 2001]. Also, they may induce rectal mucosal inflammation and affect the defecation function [Borrelli 2009; Iacono 2006]. It may be speculated that while CMP may reduce motility and promote constipation in some children, with the absence of other signs of allergy constipation is not is likely to be connected to CMPA.

Infant proctitis or rectal bleeding is also traditionally associated with CMPA. Two prospective studies addressed this association: CMPA was diagnosed in 7/40 infants with hematochezia in a study done by Arvola et al. [2006], and in 14/22 infants in a study done by Xanthakos et al. [2005]. In most cases of infant proctitis, the occurrence of hematochezia is benign and self-limited and it occurs as often in exclusively breastfed as in formula-fed infants. If associated with CMPA, there will likely be a family history of atopy and concomitant (or later challenge-associated) skin manifestations.

Chronic diarrhoea may be caused by cow’s milk-allergic enteropathy [Ford 1983; Hill 1986; Host 1990; Kuitunen 1975; Savilahti 2000; Savilahti 1985]. Signs of malabsorption and a failure to thrive may develop if left untreated. The exact pathogenesis of this presentation is still being debated since, at present, research activity has focused more on the acute FPIES-like pathophysiology. FPIES might actually represent the extreme end of gut mucosal involvement in CMPA and should only be diagnosed in infants below 12 months of age [Katz 2011; Sicherer 2000a]. However, CMPA may only affect the small intestine in the form of duodenitis/jejunitis. This results in chronic diarrhoea and malabsorption. Histologically, there is often villous atrophy and intraepithelial lymphocyte infiltrations (and occasionally eosinophilia) [Savilahti 2000]. ‘Allergic gastroenteritis/enterocolitis’ is a term used in the existing literature mainly to refer to histologically confirmed, eosinophil-dominated inflammation, which most authors believe (often without further proof) to be allergic in nature, and which typically occurs in slightly older children and adolescents. With primary eosinophilic gastrointestinal disease, the association with food allergies is estimated to be approximately 25–75%, and according to a recent review, a controlled food challenge is in fact needed to confirm the CMPA diagnosis [Yan 2009]. Regarding only paediatric eosinophilic esophagitis, the frequency of a concurrent allergic disease (food allergy, allergic asthma or rhinitis, or atopic eczema) is 42–93% [Liacouras 2011], with frequent IgE sensitisation to both food and aeroallergens. A differential diagnosis of eosinophilic (entero-)colitis includes intestinal infections and inflammatory bowel disease as well as food allergies. Eosinophilic gastroenteritis has a fairly favourable prognosis, even without interventions [Pineton De Chambrun 2011], but eosinophilic esophagitis may progress to a stricturing fibrotic esophagitis.
as the years progress [Debrosse 2008]. In infant proctocolitis, the association with food allergies is not evident, even if rectal biopsies are dominated by eosinophilic inflammation, as proven by a controlled trial [Arvola 2006].

CMPA is commonly mislabelled in children below 12 months of age. Elizur et al. [2013] presented data from a prospective population-based follow-up study. Researchers identified a false diagnosis of CMPA in 2.8% of the infants born during the study period. For those infants, the most frequently reported symptoms were skin rashes (44%), vomiting (21.5%) and diarrhoea (18.5%). Colic was reported for only 4% of the infants, while restlessness was reported for 11.8% of them. The median age of onset was two months [Elizur et al. 2013]. In addition to parental diet manipulations, iatrogenic, physician-initiated diet restrictions also contribute to this phenomenon [Boehm 1998; Cannioto 2010; Eggesbo 2001]. Parents generally follow diets prescribed to their child by a doctor meticulously [Tuokkola 2010]. Restricted diets as well as single nutrient deficiencies may stunt an infant’s growth and cause the infant to be underweight [Aldamiz-Echevarria 2008; Boehm 1998; Christie 2002]. The frequency of parental perceptions of adverse reactions to CM is remarkable [Eggesbo 2001; Pyrhonen 2009; Venter 2006]: such reactions are noted by parents in as many as 12% of infants below the age of 12 months. With the established incidence of CMPA in early childhood at 1–2% or less, the likelihood of mislabelling the child as CM allergic is considerable if relying on patient history alone, without employing proper diagnostic procedures.

2.4.2 The diagnosis of GI-CMPA

CMPA is diagnosed using the oral food challenge. There are only two instances when it can be directly omitted: 1) in the case of a history of immediate mucocutaneous food allergy symptoms after an anaphylactic reaction [Boyce 2010], or 2) in the case of a young FPIES patient with a previous history of severe acute systemic reactions (hypotension and repetitive vomiting within 24 hours of ingesting cow’s milk protein, requiring hospitalisation) [Sicherer 2000a]. Specific IgE and SPT levels may guide the clinician in the diagnostic process, but according to three recent guidelines, a food allergy diagnosis can never be based on laboratory testing alone [Boyce 2010; Fiocchi 2010; Sackeyfio 2011].

The gold standard for making a diagnosis is the DBPCFC [Bock 1990; Koletzko 2012; Sampson 2012]. Several authors have described its use and accuracy compared with the open OFC. The reported proportions of positive DBPCFCs are 28.8–34.4% in patients with suspected delayed-onset CMPA (in both atopic dermatitis and gastrointestinal manifested CMPA) [Baehler 1996; Dambacher 2013; Gellerstedt
Venter et al. [2007] compared the open FC and the DBPCFC in patients with any of the same food allergies. They performed 41 DBPCFCs, 22 of which (53%) were positive. In another population-based study on the prevalence of cow’s milk allergy by Venter et al. [2006], the rates for positive cow’s milk challenge outcomes were significantly smaller when using the DBPCFC: 2.3% of patients reacted to CMP in the open OFC and only 1.0% in the DBPCFC. The above-mentioned studies also reported that significant placebo reactions occurred at rates of 15–20%. Among children suspected of CMPA, the proportion of negative food challenges is >80% if CM-specific IgE is below 0.53 kU/l [Beigelman 2012]. Parental acceptance of the DBPCFC protocol and results is good [Käila 1997; Venter 2007]. In conclusion, the use of open OFC is not based on evidence when diagnosing GI-CMPA without immediate, FPIES-like severe symptoms.

A gastrointestinal manifesting food allergy (in the absence of major mucocutaneous symptoms) is seldom mediated by IgE, especially when it is FPIES [Ford 1983; Hill 1986; Host 1998; Sicherer 2005]. The prevalence rates of IgE positivity in GI-CMPA are not available, but the overall rate of CMPA was 61% in patients with a positive SPT for CM (≥3 mm) and 45% for elevated CMP-specific IgE among a prospective population-based CMPA cohort in Finland [Saarinen 1999]. In another Finnish study on 304 infants with CMPA (34% with GI symptoms), none of the delayed-type CMPA patients had either positive SPTs or IgE for CM [Vanto 1999]. This category included patients with atopic eczema and gastrointestinal symptoms. Atopic patch testing (APT) is often mentioned within the context of delayed-type CMPA diagnostic procedures. However, there is clear evidence that the results from APT concur with those from SPT since the negative and positive predictive values for positive OFC are only at 0.52 and 0.63, respectively [Majamaa 1999; Saarinen 2001; Vanto 1999]. One plausible explanation for the futility of APT in GI-CMPA is the distinct differences in the phenotypes of cutaneous, circulating and intestinal lymphocytes [Caubet 2011]. In light of the poor predictive values, performing the APT for CM would not render the DBPCFC unnecessary in GI-CMPA, and therefore, the APT cannot be recommended for diagnosing GI-CMPA.

The DBPCFC

The DBPCFC protocols described in the literature vary, especially with respect to the dosing and blinding methods [Vlieg-Boerstra 2004]. The following discussion pertains to the DBPCFCs used to diagnose a delayed-type FA. The elimination period preceding the challenge should be a minimum of two weeks [Bock 1990; Niggemann 2007a; Sicherer 1999]. This allows for the presumed immunological activation from the quiescent state. A positive elimination response is noted if the patient is rendered symptom free. The asymptomatic status is a prerequisite to performing the food challenges. If the established elimination diet is not helpful, the
suspicious food is reintroduced and the diagnostic work-up continued in other directions. Bock et al. first attempted to standardise the DBPCFC in 1988 [Bock 1988], but the protocols used and those that have been published since are not uniform. CMP (and a corresponding placebo) has been given as a powder [Baehler 1996; Majamaa 1999; Sicherer 2000b], as capsules [Bock 1988] or as regular infant formula [Vanto 1999]. Regular infant formula has the benefit of most closely resembling real-life CMP consumption, but it has been criticized because it may also elicit symptoms related to components of CM other than protein, such as lactose. The doses used by various authors have been somewhat different. Many researchers aim to find the minimum eliciting dose, and thus use protocols starting with minuscule amounts of CMP and increase the doses at intervals of 15–30 minutes [Niggemann 2007a]. Such an approach is reasonable for immediate-reaction (IgE-positive) CMPA, but there is no evidence of its usefulness in patients with a history suggestive of the delayed-type CMPA. Since symptoms are elicited usually within 24–72 hours of ingestion among the latter type of patients, it is more important to continue the challenge for a longer period of time (5–7 days) [Isolauri 1996; Venter 2007], and to carefully take into account the possibility of confounding factors, such as infections.

Some authors claim that the negative 3–5 day DBPCFC might be too short to elicit the delayed type of gastrointestinal symptoms, which in some children take up to 1–2 weeks to develop [Hill 1995]. This suggestion has not been methodologically studied and remains unproven; the overwhelming body of literature reports that the timeframe between allergen ingestion and FA symptoms should be within 24–72 hours. Each challenge, both the active and the placebo challenge, should only be conducted if the child is well and infection free.

The results of the DBPCFC are not 100% conclusive either way, as several confounding variables exist; it is better to discuss the possibility of both placebo reactions and false-positive reactions prior to the challenges [Caffarelli 2001; Gellerstedt 2007; Niggemann 2007b]. Possible reasons for a false-negative DBPCFC are few, but concomitant drug use could affect the result and the use of cooked food may mask the patient’s reactivity to raw antigens [Eigenmann 2000]. In terms of the protocol for increasing the dose, there may be a delayed influx of pro-inflammatory mediators [Caffarelli 2001], which would cause the reaction to occur only after 5–7 days.

2.4.3 The Treatment of GI-CMPA

Strictly speaking, it has not been proven that eliminating CMP will in fact cure CMPA [Boyce 2010], even though at present it is the only management option for reducing the symptoms. There is a widespread consensus in the literature that for children
with a past history of anaphylaxis and IgE-mediated CMPA, total CMP elimination is warranted [Venter 2012]. The same applies to patients with severe FPIES reactions [Sicherer 2005]. However, recent research on tolerance acquisition has created a debate over the need to totally eliminate CMP in those patients whose symptoms are less severe [Dupont 2013; Netting 2013]. There is evidence of CMP elimination (among those with previously milder symptoms of IgE-mediated CMPA) actually resulting in anaphylaxis [Flinterman 2006]. There are no randomised controlled trials addressing the effect and optimal duration of CMP elimination among GI-CMPA patients. Studies that report the age at which CMPA patients tolerate CMP only include patients with previous strict CMP elimination. Since CMPA elimination likely affects the immune processes involved in tolerance acquisition [Wisniewski 2013], it is possible that the reported timeframes are in fact biased and may overestimate the amount of time it takes to develop a tolerance to CMPA. Also, the differences between IgE-mediated CMPA and non-IgE CMPA should be taken into account. In fact, a recent recommendation suggested a more active and faster protocol for introducing CMPA, especially for GI-CMPA patients [Dupont 2013].

Specific oral tolerance induction has been gradually showing more evidence of positively affecting the treatment of IgE-mediated CMPA, but clinical guidelines are needed for its use beyond research purposes [Yeung 2012]. The use of specific tolerance induction protocols have not been reported for GI-CMPA patients. Tolerance to baked/cooked CM is frequent, and using these products (at least in small amounts) is recommended because they promote more rapid CMP tolerance [Kim 2011]. The same applies to fully matured cheese, specifically for non-IgE CMPA patients [Alessandri 2012]. Freezing fresh food does not seem to alter its allergenicity [Ziegert 2007].

2.4.4 The Prognosis for GI-CMPA

The prognosis for non-IgE mediated CMPA is in general good: there is a nearly 100% rate of CMP tolerance at the age of 3 years [Saarinen 2005]. Despite the alarming early symptoms, the prognosis for outgrowing FPIES is especially positive and controlled food challenges are recommended at least yearly [Hwang 2009]. Nearly 100% of FPIES patients are symptom free at two years of age [Hwang 2009; Sicherer 2000a]. As discussed above, there is the possibility that the amount of time it takes to gain a tolerance to CMP will be overestimated. However, compared to the 60% tolerance rate at three years of age in IgE-mediated CMPA patients [Saarinen 2005], the prognosis for outgrowing CMPA in the absence of cow’s milk-specific IgE is favourable. Specific data on the prognosis and follow-up for GI-CMPA patients, other than regarding FPIES, are not available.
Long-term follow-up on CMPA patients (beyond achieving tolerance) is rarely reported. An early childhood CMPA diagnosis associates with school-age functional gastrointestinal disorders [Kiefte-De Jong 2010; Saps 2011] and the later diagnosis of inflammatory bowel disease [Virta 2013].
2.5 Lactose-induced symptoms in infancy

Human breast milk has an abundant amount of lactose (up to 7 g/100 ml) [Khan 2013]. Infant intestinal epithelial cells in the small intestine express lactase enzymes, the expression of which is upregulated in response to feeding [Shulman 2005], with significantly lower levels in premature infants and maximum levels reached only during the last trimester [Shulman 1998; Weaver 1986]. With congenital lactase deficiency, there is profound watery diarrhoea starting immediately after enteral feedings commence. Congenital lactase deficiency is more frequent in the Finnish population compared to other nationalities, with an incidence of 1:60 000 and with several different genetic mutations having been identified [Torniainen 2009]. Levels of the constitutively regulated lactase enzyme activity decrease slowly during childhood. A mutation in the lactase gene regulation area (CT-13910) is responsible for the persistent lactase enzyme activity after early childhood [Rasinpera 2004]. This mutation has become enriched in dairy-dependent populations, such as among the Finnish people. The congenital-type lactase deficiency mutations affect the lactase enzyme protein structure itself, whereas the lactase enzyme activity can be reduced for patients with the CT-13910 mutation by decreasing the messenger RNA regulating enzyme activity. Since the CT-13910 region may be involved in other regulatory functions as well, the full extent of the adult-type hypolactasia genotype CT 13910 is unknown [Järvelä 2009].

Lactase promotes lactobacilli and bifidobacteria in infant gut microbiota, which may be beneficiary for term infant because it enhances the switch to TH1-mediated immunity [Francavilla 2012]. However, an overload of lactose in the intestinal lumen may ensue in premature infants with low levels of lactase activity, which may then promote an overgrowth of small intestinal bacteria, the production of harmful SCFA and even the risk of NEC [Shulman 2005]. Lactose malabsorption (as measured through breath hydrogen testing) sometimes occurs in colicky infants [Infante 2011; Miller 1989; Moore 1988]; hence, a formula containing low lactose levels may be beneficial for reducing the crying time, although a randomised, placebo-controlled study is needed to confirm this assumption. Lactose malabsorption was more pronounced in infants whose birth weight was less than 2.5 kg [Miller 1989]. According to Schulman et al. [2005], the small intestinal cell mass also affects the total lactose absorption to some extent, offering a simple explanation for why the size of the infant affects lactose absorption. In adult-type hypolactasia (genotype CT13910), the decrease in lactase enzyme activity is slow and individual [Rasinpera 2005].
2.6 Psychological aspects of gastrointestinal symptoms of CMPA

2.6.1 Psychosocial adjustment to CMPA

Patients and families with food allergies experience elevated psychosocial stress, which is primarily associated with the fear of food allergy reactions [Lebovidge 2009; Marklund 2006]. There is evidence that gastrointestinal symptoms in particular entail an increased risk of psychosocial maladjustment, maybe reflecting the diagnostic challenges involved [Marklund 2006]. However, the reluctance of parents to re-introduce foods proven acceptable by the controlled OFC (even DBPCFC) is also a concern [Eigenmann 2006]. As many as 71% of those with a successful re-introduction of foods indicated significantly better quality of life afterwards [Flammarion 2010]. Maternal characteristics may play a role in this process, since when specifically asked, mothers listed a reluctance to face new challenges and a fear of losing control as reasons for not re-introducing food to the child [Strinnholm 2010]. Studies addressing psychosocial aspects related to FA mainly focus on the IgE-mediated disease; there are even fewer studies that address this topic in food-allergic/symptomatic infants.

Meldrum et al. [2012] studied a large cohort (n=324) of children considered at risk for allergic disease (based on a maternal history of allergic disease). Of this number, 29 were diagnosed with non-IgE allergy; compared to the non-allergic children, these 29 children had significantly higher scores (as measured by the Child Behaviour Checklist by Aachenbach) for internalising behaviour problems at 18 months of age. They did not detect such corollaries among the IgE-mediated allergy patients. Their diagnosis of a non-IgE food allergy was not based on the DBPCFC, which is an important confounding variable, as discussed before in this text. The question remains then, does non-IgE FA predispose infants and young children to later neuro-developmental issues, or is there a common origin for the both? In other words, could it be possible that the symptoms interpreted as non-IgE FA actually stem from existing psycho-developmental problems, such as those discussed below?

2.6.2 Infant feeding disorders

Feeding disorders (FDs) in early childhood are increasingly being studied by scholars [Bryant-Waugh 2010; Manikam 2000]. The term feeding disorder is not
interchangeable with eating disorder (with the latter being seen in older children and adults), as it emphasises the dyadic nature of feeding in infancy [Chatoor 2002]. The Chatoor classification subclasses are of special interest with respect to the scope of the present literature review: 1) infantile anorexia and 2) an FD associated with a concurrent medical condition.

Infantile anorexia refers to an FD where the infant apparently loses interest in eating at the time when transition to the developmental period of separation and individuation occurs. This type of FD is characterised by dyadic conflict, less dyadic reciprocity and maternal contingency, all of which affect the feeding sessions and result in increasing difficulties in the feeding situations themselves, with the subsequent possibility of mutual frustration and even force-feeding. Such infants are often perceived by their mother as being temperamentally difficult [Chatoor 2000]. Others have chosen to refer to this condition as transitional FD [Levy 2009].

Some organic medical conditions predispose infants to an FD, with the key problems being GI symptoms associated with feeding (nausea, vomiting, abdominal pain), but diseases leading to respiratory distress associated with feeding (including chest and cardiac disease) may also trigger similar responses [Manikam 2000]. The infant may develop a strong association of unpleasantness with feeding, resulting in anticipatory gagging, food refusal, head turning and vomiting. Co-operation between the different disciplines is needed to decipher the extent of the organic disease and the psychological sequelae [Bryant-Waugh 2010; Chatoor 2002; Levine 2011; Levy 2009]. The clinical presentation of FDs offers few clues as to the causal origins. Vomiting was just as frequent (in the cohort described by Levy et al.) in the ‘non-organic’ FD group as in the organic-onset group. The group labelled non-organic often included an identifiable organic trigger in the patient’s history, even though such a trigger was no longer present, emphasising the need to assess the patient’s history carefully. Nutritional deficiencies (caloric or specific nutrients) are common [Lindberg 2006].

The major differentiating symptoms between the organic (ongoing disease) and non-organic (psychological and/or organic history) groups were abnormal feeding practices (nocturnal feeding, excessive distraction or stimulation during feeding and forced feeding), anticipatory or/and feeding-related gagging, and food refusal (incl. head turning), all of which were indicative of a presently non-organic FD [Levine 2011; Levy 2009]. Proposed new FD criteria for better identifying FD patients with a potential for successful psychological intervention, known as the Wolfson criteria [Levine 2011], are listed in Table 3.

These criteria make it possible for both organic and non-organic FD to co-exist if the feeding-related symptoms continue after proper medical treatment of the underlying disease. An ongoing organic disease is present in 18–35% of FD patients [Levy 2009], emphasising the need to also include the psychological aspects of care for
suspected organic-onset FD if signs of FD develop. It is a point of concern that older age at the time of referral and younger age at the onset of symptoms were both significant factors in treatment failure, implicating a need for earlier recognition of FDs [Levine 2011].

<table>
<thead>
<tr>
<th>The Wolfson criteria</th>
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</thead>
<tbody>
<tr>
<td>1) a. The gagging (or vomiting) reflex associated with feeding; OR</td>
</tr>
<tr>
<td>b. The presence of abnormal feeding situations and practices (extensive distraction, nocturnal feeding, time-consuming feeding), AND</td>
</tr>
<tr>
<td>2) Absence of an obvious organic disease, or lack of response to appropriate medical treatment of the organic disease, AND</td>
</tr>
<tr>
<td>3) The start of symptoms age &lt; 2 years, AND</td>
</tr>
<tr>
<td>4) Persistent food refusal lasting &gt; 1 month</td>
</tr>
</tbody>
</table>

**Table 3.** The Wolfson criteria (modified from [Levine 2011]) for diagnosing infant feeding disorders.

Maternal characteristics also affect the development of FD [Ammaniti 2010; Farrow 2006; Wright 2006]. Maternal anxiety in particular, but also maternal depression and reduced interpersonal sensitivity, have been found to alter the emotional interaction between mother and child; this then promotes infantile FD [Gueron-Sela 2011]. Maternal anxiety may be triggered by worrying about the child's weight [Gueron-Sela 2011], but it may also be a pre-existing maternal characteristic [Ammaniti 2010]. FD has also been associated with delayed speech and language development [Fabrizi 2010; Manikam 2000].

### 2.6.3 The association of feeding disorders with gastrointestinal complaints in infancy

The major organic clinical conditions predisposing children to FD include GERD and food allergies [Levine 2011], although systemic studies addressing how these conditions pertain to food allergies are lacking. Feeding problems and even FD are common in patients with GERD [Dellert 1993; Mathisen 1999], affecting up to 28.5% of them [Karacetin 2011]. GERD patients may have impaired swallowing functions, but not all of the feeding-related challenges are due to physical factors. As discussed
above, FD in general may be triggered by an organic or traumatic event and associate with maternal behavioural qualities. For GERD patients, it has been shown that maternal anxiety mediates the development of FD [Karacetin 2011]. Such studies have not been done for food allergies, however, even though the connection has been recognised [Wang 2010]. The occurrence of FD was recently estimated at 16.5% for patients with eosinophilic esophagitis [Mukkada 2010], with the majority of them requiring psychological intervention. Concern has been raised over paediatricians treating infants with reflux symptoms or colicky crying and concentrating on the somatic treatments only, while overlooking the possibility of co-existent non-organic feeding problems [Douglas 2013]. Using nasogastric tubes to feed infants with an FD may in fact make it worse [Haas 2009]. Infant behavioural characteristics also have a significant impact on GERD-associated FDs: mothers of GERD patients with an FD frequently rated their child as more difficult and demanding [Mathisen 1999].

2.6.4 Parenting stress and parentally perceived child characteristics evident in gastrointestinal diseases during early childhood

The Parenting Stress Index (PSI) is a technique developed to measure the amount of stress within the parent-child system [Loyd 1985]. It was developed to help recognise individual parent-child systems under stress at an earlier point in time. It is based on three major domains of stressors: child characteristics, parent characteristics and situational life stress. The PSI has been widely used in various clinical conditions and diseases [Abidin 1995]. The total Parenting Stress was abnormally high in infants with eosinophilic esophagitis [Wu 2012], as well as in colicky infants expressing GER symptoms [Miller-Loncar 2004]. The PSI Child Domain scores were significantly elevated in infants with a clinical FD [Martin 2013], whereas this score (as well as the total PSI) was within the normal range in children needing a feeding gastrostomy for any reason [Avitsland 2012]. Parenting stress increased significantly when parents perceived that the infant had sleeping problems, even when this was not verified through diaries [Sinai 2012]. The subscale Child Domain assesses child characteristics, including temperament characteristics. It also incorporates into each question the effect a given temperamental factor has on the parent. High scores in the PSI Child Domain reflect children who have behavioural qualities that may impede successful parenting [Hanson 1990].

Infant temperament and behavioural characteristics can be measured using various tools. All of the tools have been criticised for a lack of objectivity, as the questionnaires merely measure the parents’ perceptions. Infant difficult temperament has been linked to, e.g. feeding disorders [Farrow 2006], fussy behaviour and sleep disturbances [Hayes 2011], colicky crying [Lehtonen 1994] and
later psychiatric problems in adolescence [Teerikangas 1998]. The Infant Temperament Questionnaire (ITQ) [Carey 1970, 1978] is a 71-item measure for assessing characteristics of an infant’s temperament. Martin et al. [1997] identified five specific temperament factors in the ITQ: biological irregularity, threshold to stimuli, distress to novelty, activity/intensity, and fussy/demanding. Regarding the use of questionnaires to assess infant temperament, there is a significant correlation with maternal characteristics and the infant’s perceived temperament [Mäntymaa 2006], which may even be more substantial than the actual infant temperament traits themselves [Vaughn 1987]. In addition to maternal anxiety, another factor affecting a parent’s perceptions of a child’s difficult behaviour has to do with a mismatch in the parent-child temperament [Carey 1998]. Regardless of the origin of the parental perceptions, a finding indicating a difficult temperament may reflect a potentially problematic mother–child interaction and should be acknowledged [Keenan 1998; Mäntymaa 2006].

2.6.5 Mother–child interaction and emotional availability

Mother-child interaction has been widely studied. One specific methodology is the study of emotional availability (EA) [Biringen 2000]. The emotional availability of mothers in mother–child interactions is systematically linked to positive child outcomes in terms of self-regulation, sleeping patterns, secure attachment and socialisation skills [Lehman 2002; Little 2005; Scher 2001]. Low levels of maternal emotional availability have, in turn, been associated with early childhood psychosocial problems and attachment insecurity [Ziv 2000]. As discussed above, low levels of dyadic emotional availability were found in children suffering from FDs, indicating that mother–child interactions and maternal anxiety play a major role in the development of FD [Ammaniti 2010; Gueron-Sela 2011; Wiefel 2005]. The direction of effects, however, is not easy to trace, since parent-child interaction is a dynamic reciprocal system [Fiese 1989].

The Emotional Availability Scales (EAS), a measure developed to depict the quality of parent-child interaction, consist of six dimensions addressing various areas of emotional interaction between the child and mother [Abidin 1995] (see Table 4). With FDs, mother–child dyads express less maternal sensitivity and more intrusiveness, as well as less child responsiveness and involvement of the mother. Maternal anxiety is the mediating factor affecting the problematic emotional interaction in FD patients [Gueron-Sela 2011]. Thus, maternal worry (triggered and amplified by the child’s weight/feeding habits) may lead to difficulties in reading the child’s interactional cues, e.g. offering food when the child is not ready for feeding.
This intrusiveness may in turn lead to the child withdrawing from interaction and also refusing food. The child’s behaviour then plausibly causes increasing levels of maternal anxiety. The EAS has not been previously studied for patients with CMPA.

<table>
<thead>
<tr>
<th>The EAS dimension</th>
<th>Explanation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mother</strong></td>
<td></td>
</tr>
<tr>
<td>Sensitivity</td>
<td>Relates to a mother’s positive affect towards the child and her responsiveness to and acceptance of the child. It also incorporates maternal awareness of the infant’s cues and appropriate responsiveness to them.</td>
</tr>
<tr>
<td>Structuring</td>
<td>Refers to the mother’s way of structuring or scaffolding the child’s environment and play.</td>
</tr>
<tr>
<td>Non-intrusiveness</td>
<td>Refers to the degree to which the mother is available without interfering with the infant’s autonomy and space.</td>
</tr>
<tr>
<td>Non-hostility</td>
<td>Evaluates maternal behaviour that is free of impatience, harshness or malice</td>
</tr>
<tr>
<td><strong>Child</strong></td>
<td></td>
</tr>
<tr>
<td>Responsiveness</td>
<td>Evaluates how the child responds to maternal cues and expressions</td>
</tr>
<tr>
<td>Involvement</td>
<td>Refers to the degree to which the infant wants to interact with the mother.</td>
</tr>
</tbody>
</table>

*Table 4.* The dimensions of the Emotional Availability Scales (EAS) attributed to the mother and the child.
3 Aims of the study

The aim of this thesis was to study factors associated with the diagnosis of gastrointestinally manifesting cow’s milk protein allergy.

The specific aims were as follows:

I. To characterise the symptoms and clinical findings associated with gastrointestinally manifesting cow’s milk protein allergy using the double-blind, placebo-controlled food challenge as a reference;

II. To assess the value of measuring the immunological markers in stool and cow’s milk-specific immunoglobulins in serum of patients suspected of GI-CMPA;

III. To investigate parental perceptions of a child's behavioural characteristics in patients suspected of GI-CMPA;

IV. To evaluate the mother–child emotional interaction in children suspected of GI-CMPA using a validated qualitative interaction measure, the Emotional Availability Scales.
4 Subjects and methods

4.1 Patients

For this prospective research study, infants and young children below 4 years of age were recruited who had been referred to the Helsinki University Hospital Pediatric Allergy Unit or Children’s Hospital between January 2010 and January 2011 for gastrointestinal symptoms suspected of CMPA. All children were either already following a CM-free diet (implemented by the referring physician or the parents themselves) or were instructed to start a CM-free diet according to instructions sent from the Allergy Unit. Only those responding to the CM-free diet were considered eligible for the study. Patients with skin symptoms were not excluded so long as the major complaint was gastrointestinal. Nor were patients excluded on the basis of cow’s milk-specific skin prick tests or IgE in cases where these tests had been performed by the referring physician. Patients with diagnosed chronic diseases were excluded as were those who not respond to a CM-free diet. The flow chart in Figure 2 depicts the patient recruitment process, patient numbers and study timelines. Table 7 (page 51) shows the cohort details.

For sub-study IV, a subgroup consisting of 28 (49%) patients (and mothers) was invited to participate in a video recording session depicting a free-play session with the mother and child. The patients were chosen randomly on the basis of predetermined alternate days when the video recording equipment was available.
4.2 Controls

Control patients were recruited from patients 0–4 years of age attending the Pediatric Allergy Unit for any disease other than atopic eczema, FA or another atopic/allergic disease or suspicion thereof. The most usual diagnoses were suspected reactions to antibiotics and non-atopic wheezing. The mean age of the control patients was 13.2 months (range 4.8–30.0).

Concerning the EAS, the study patients’ results were compared to national normative data derived from a previous research project [Salo 2010], as discussed below.
4.3 Methods

4.3.1 The double-blind, placebo-controlled food challenge for cow’s milk

The DBPCFC protocol is outlined in Figure 3. A detailed description of the protocol is presented in sub-study (I). The challenge cycles lasted five days each. The milk used for the placebo challenge consisted of a breast-milk substitute formula the patient was perceived (by the parents) to tolerate well during the elimination diet period. The milk used for the provocation challenge was prepared by mixing the placebo formula (ratio 2:1) with standard infant cow’s milk formula. The parents kept a detailed diary of the child’s symptoms throughout the challenge. A minimum of one week of symptom-free time between the challenges was observed. The parental perceptions of the child’s symptoms during each challenge cycle formed the basis for the eventual CMPA diagnosis. The challenge was deemed positive only if significant symptoms similar to those suspected previously occurred during the provocation milk challenge.

![Figure 3. The DBPCFC challenge protocol.](image)

*Active challenge = CMP provocation cycle*

In the case of a negative challenge (no symptoms or similar symptoms during both challenges), a mix of 1:1 hypoallergenic formula and CM formula was recommended during the first weeks after the challenge. The challenge-positive patients were instructed to keep following the CM elimination diet. Since none had immediate
severe symptoms during the DBPCFC, re-challenges were recommended at home with small amounts of cow’s milk protein every 3 months.

4.3.2 Laboratory measures

The following laboratory tests were determined using routine, commercially available methods: full blood counts, serum cow’s milk-specific IgE test (Phadiatop inc.), total serum IgA blood test (semi-quantitative EIA, detection limit 0.1 g/l), stool occult blood test (Actim®, sensitivity 40 µl blood/100 g stool), lactase CC13910 genotyping test (cyclic minisequencing) and a serum high-sensitivity C-reactive protein test (hs-CRP; Roche Diagnostics, detection limit 0.05 mg/l).

SPTs were carried out on the volar aspect of the forearm with a positive control (hista-mine hydrochloride 10 mg/ml, ALK-Abello), a negative control (buffer solution, ALK-Abello) and a CM formula (NAN, Nestle Finland). The SPTs were read after 15 minutes. The weal’s longest and shortest perpendicular axis was measured, and the results were expressed as the mean weal diameter in millimetres. Reactions with a mean weal diameter of 3 mm or larger were considered positive [Boyce 2010].

**Measurement of CM and ovalbumin-specific antibodies**

Serum antibodies of IgA, IgG, IgG1 and IgG4 for cow’s milk β-lactoglobulin and α-casein and ovalbumin were analysed using enzyme-linked immunosorbent assays (ELISA). The results are given as Arbitrary Units (AU), with normative levels based on previous reports for the antigen-specific IgA and IgG [Savilahti 1993], as well as for the antigen-specific IgG4 and IgG1 [Savilahti 2010a]. The Arbitrary Units (AU) were derived from the optical densities of the reference serum curve with a high level of antibodies after subtracting the blanks. The reference serum was a pool of sera collected for IgA and IgG isotypes and its concentration was decreed to be 100 AU. The detection limits (expressed as AU) were as follows: α-casein-specific IgA 0.26; IgG 0.70; IgG1 0.28; IgG4 0.28; β-lactoglobulin-specific IgA 0.3; IgG 0.028; IgG1 0.028; IgG4 0.028; ovalbumin-specific IgA 1.5; IgG 0.26; IgG1 1.3; and IgG4 5.20. Values below the detection limit were entered as the detection limit divided by two.
**Measurement of faecal β-defensin 2, faecal IgA, and faecal calprotectin**

Faecal calprotectin was measured using the PhiCal Test (Calpro AS, Oslo, Norway; NovaTec Immunodiagnostica, Dietzenbach, GmBH, Germany). Before analysing faecal β-defensin 2 (F-BDEF) and Immunoglobulin A (F-IgA), the thawed samples were homogenised in a phosphate-saline buffer and centrifuged for 15 min at 10 000 g, +4°C, to retrieve a supernatant for the measurements. Enzyme-linked immunosorbent assay (ELISA) was used according to the manufacturers’ instructions for measuring faecal levels of human β-defensin 2 (Immundiagnostik, Bensheim, Germany; detection limit 0.077 ng/mL). Faecal IgA was analysed using ELISA based on the previously reported method [Saarinen 2002]. The detection limit for F-IgA was 5 μg IgA/l.

**4.3.3 Measures of child behavioural characteristics**

**The Parenting Stress Index Child Domain**

The Parenting Stress Index (PSI) [Abidin 1995] has been developed as a tool to identify and quantify the stressors associated with parenting. Regarding the child’s characteristics, the development and validation process identified four temperament-related variables as well as two other factors related to parental perceptions about parenting a particular child (Table 5). The PSI incorporates in each question the effect that a given temperamental factor has on the parent. The subscales are further discussed below. All of the study participants’ mothers and controls’ mothers were asked to return the questionnaires before the food challenge (or, in the case of the control patients, during their outpatient clinic appointment only) and at the six-month follow-up.

The PSI Child Domain has a total of 47 items divided into six subscales. Each item is rated on a five-point Likert scale ranging from (1) ‘strongly disagree’ to (5) ‘strongly agree’. The range of points is 50–145. Increased scores are associated with more stress.
### The PSI Child Domain

<table>
<thead>
<tr>
<th><strong>Adaptability</strong></th>
<th>Addresses the difficulties the child has regarding change and transitions.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Demandingness</strong></td>
<td>Relates to the pressure the child perceivably lays on the parent: examples include open defiance, aggression, intrusions and requests for attention and service.</td>
</tr>
<tr>
<td><strong>Mood</strong></td>
<td>Refers to stressors the parent may perceive as anxiety or anger provoking (excessive crying, withdrawal, depression).</td>
</tr>
<tr>
<td><strong>Distractability/Hyperactivity</strong></td>
<td>Refers to stressors associated with an increased need for parental vigilance and active parental management.</td>
</tr>
<tr>
<td><strong>Acceptability</strong></td>
<td>Relates to the child’s characteristics associated with social desirability.</td>
</tr>
<tr>
<td><strong>Reinforces Parent</strong></td>
<td>Deals with the components of the bonding process between the parent and child.</td>
</tr>
</tbody>
</table>

**Table 5.** The Parenting Stress Index (PSI) Child Domain.

### The Infant Temperament Questionnaire

Mothers of all the study participants and controls below the age of 18 months were asked to complete The Infant Temperament Questionnaire (ITQ) [Carey 1978] for the study patients both before the DBPCFC and at the six-month follow-up. The controls’ mothers returned the questionnaire during their outpatient clinic appointment. The ITQ comprises 71 items that assess infant temperament characteristics. **Table 6** depicts the contents of the ITQ.

A new factor labelled ‘Difficultness’ was used in the present study; it combines the subscales Biological irregularity, Fussy/demanding and Distress to novelty. It includes 26 items. These items address various aspects of an infant’s life outside gastrointestinal- or feeding-related issues. Each item is scored on a scale of 0 – 1 – 2. The ITQ difficultness score ranges from 0 to 52. Elevated scores indicate a more difficult temperament.
### The ITQ subscales

<table>
<thead>
<tr>
<th>Subscale</th>
<th>Content</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Biological irregularity</strong></td>
<td>How regular is the child in her or his daily functions (falling asleep, waking, eating, defecating, etc.)?</td>
</tr>
<tr>
<td><strong>Fussy/demanding</strong></td>
<td>What is the child’s general mood like? How does the child respond to grooming, changing diapers, etc.? How does the child like playing/being alone?</td>
</tr>
<tr>
<td><strong>Distress to novelty</strong></td>
<td>How does the child react to new places/people/food?</td>
</tr>
<tr>
<td><strong>Threshold</strong></td>
<td>What is the threshold for reacting to discomfort?</td>
</tr>
<tr>
<td><strong>Activity/intensity</strong></td>
<td>Physical activity (moving) during various situations, and its level of intensity.</td>
</tr>
</tbody>
</table>

*Table 6. The subscales of the Infant Temperament Questionnaire (ITQ) (the subscales included in the difficulty factor are marked with *).*

### 4.3.4 The Emotional Availability Scales

**The Emotional Availability Scales (EAS) [Abidin 1995]**

The EAS (4th edition, original US version) was used for this assessment. The EAS includes four maternal scales (Sensitivity, Structuring, Non-intrusiveness and Non-hostility) and two child scales (Responsiveness to Mother and Involvement of Mother), which are evaluated using a 7-point scale (1–7). A more detailed description was provided on page 46. The EAS employs a holistic approach to analysing mother–child interaction, which means that the observer rates the appropriateness of behaviours, applies clinical sensitivity to interpret the emotional signalling (cues) between the mother and the child, and takes into account the context in which the interaction occurs. Thus, the EAS gives a qualitative measure of the emotional availability within the studied relationship.

**Emotional Availability Assessment**

The mother–child dyads were assessed based on the videotaped sessions of mother-infant free play lasting 15–20 minutes. The parents were instructed to spend time
and play with their children as they usually would with the following toys: mirror, ball, book, building blocks, doll (with milk bottle) and a wooden baby puzzle.

A trained rater analysed the videotapes. The inter-rater reliability of the videotapes was confirmed for 13% of the tapes by another trained rater. The inter-rater reliabilities (r) of the analyses ranged from .87 to .90 between the two raters.

The EAS Normative Data were derived from a recent study conducted in southern Finland [Salo 2010]. The EAS raters were the same in both studies. Healthy infants were recruited from well-baby clinics in two cities (Turku and Helsinki), where the clinic nurse invited every third mother and a suitably old infant to participate in the video session until a predetermined number of children per clinic had been recruited. The mean age of the infants was 9.96 months, +/- SD 2.01.

4.3.5 Statistical analyses

Mann-Whitney’s u-test was used to analyse the continuous variables. Unless otherwise specified, the continuous laboratory parameters are reported as geometric means (together with the 95% confidence interval of the mean). Either ANOVA one-way analysis of the variance or the Kruskall-Wallis test was employed for comparisons involving three groups. Categorical variables were analysed using the $\chi^2$ test (or Fisher’s exact test when appropriate). When comparing the observed frequencies against the known population prevalence, the proportional $\chi^2$ test was employed. All statistical calculations were made using the GraphPad Prism, version 5.0 for Mac (California, USA).

4.3.6 Ethical considerations

This study recruited patients who had been referred to the hospital outpatient clinic for GI symptoms suspected of CMPA. Our clinic uses the DBPCFC as a routine method for diagnosing non-IgE CMPA. The laboratory tests and questionnaires as well as the videotaping in the sub-cohort were done purely for research purposes. Information about the study was given to the parents first by phone (research nurse), and if the parents expressed interest in the study, then the study nurse sent home the documents with detailed information. Children were only enrolled in the study if both legal guardians gave their written consent to participate. The Helsinki University Hospital Ethics Committee approved this study (No.317/13/03/03/2009).
5 Results

5.1 Clinical features of children suspected of CMPA with gastrointestinal symptoms (I)

In total, 68 eligible children participated in the study, 57 of whom completed the study protocol. The median age of the patients was 8.7 months (range 2.4–40.8), with similar age distributions in the challenge-negative and challenge-positive patients (Table 7). The DBPCFC outcome is shown in Figure 3.

![Figure 3](image_url)

**Figure 3.** The DBPCFC outcome and patient numbers.

The median duration of the CM-free diet preceding the diagnostic challenge was 2.5 months (0.5–35) for both groups. According to parental recall at the time of the DBPCFC, the median age when the first symptoms were suspected was
approximately 3 months (0.5–12) for all patients and the challenge-negative group, whereas the challenge-positive children were slightly younger (median age 1 month [0.5–12], p=0.0342) when the symptoms first started (unpublished data). The eleven children who withdrew from the study had similar background data: the age range was 2.7–16 months and 5 of the 11 participants were boys (unpublished data).

<table>
<thead>
<tr>
<th></th>
<th>DBPCFC negative (n=39)</th>
<th>DBPCFC positive (n=18)</th>
<th>p-value</th>
<th>Finland 2010</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, in months (median, range)</td>
<td>8.7 (2.5–25.6)</td>
<td>8.4 (2.4–40.8)</td>
<td>0.9043</td>
<td>NA</td>
</tr>
<tr>
<td>Maternal age, in years (median, range)</td>
<td>33 (22–41)</td>
<td>32.5 (25–39)</td>
<td>0.5861*</td>
<td>28.3</td>
</tr>
<tr>
<td>Maternal education college/university, n (%)</td>
<td>21 (54%)</td>
<td>6 (33%)</td>
<td>0.1680*</td>
<td>30%</td>
</tr>
<tr>
<td>Firstborns, n (%)</td>
<td>19 (46%)</td>
<td>5 (28%)</td>
<td>0.1606</td>
<td>43.7%</td>
</tr>
<tr>
<td>Parental history of atopy/allergy#</td>
<td>- Mother 23 (59%)</td>
<td>10 (56%)</td>
<td>ns</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>- Father 16 (41%)</td>
<td>8 (44%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Either 29 (74%)</td>
<td>16 (89%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Both 10 (26%)</td>
<td>3 (17%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Feeding-related problems‡</td>
<td>- Baseline 12 (31%)</td>
<td>12 (67%)</td>
<td>0.0197</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>- 6-month follow-up 6 (15%)</td>
<td>6 (33%)</td>
<td>0.165</td>
<td>NA</td>
</tr>
<tr>
<td>Use of amino acid/soy formula</td>
<td>8 / 4 (total 30%)</td>
<td>3 / 3 (total 33%)</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

**Table 7** Background characteristics of children suspected of GI-CMPA (modified from (I))

* Compared to the national numbers, the expected percentages presented here for high maternal age and education were both significantly different in the DBPCFC-negative patients (p>0.005).

# Parental atopy/allergy based on parental self-reporting of atopic eczema, asthma, food allergy or allergic rhinitis.

‡ Feeding-related problems were defined as anamnestic reports of feeding refusal, feeding-related gagging and problematic feeding practices.
The suspicion of CMPA had arisen during exclusive breastfeeding for eight infants, and their mothers had excluded CM from their diet with a perceived positive outcome in the child’s symptoms. Five of them were later proven to be CMPA negative, and three had a positive DBPCFC (unpublished data).

Maternal diet manipulations were in fact more frequent, but the retrospectively collected data regarding the extent, effect and duration of these self-imposed diet restrictions in patients with mixed breastfeeding/solids/formula was unfortunately too heterogeneous to be reliably reported. Most study participants tolerated and used hydrolysed formulas extensively with/without breastfeeding. One in every five patients was consuming amino acid-based formula (11/57, 19%), several of them as a first-line hypoallergenic formula. Soy formulas were also frequently used (7/57, 12%). However, the use of either of these formulas was not associated with an increased likelihood of a positive DBPCFC.

The DBPCFC results

Significant gastrointestinal symptoms occurred only during the active challenge in 18/57 (32%; 95% confidence interval 21–45%) patients, whereas 39 (68%) had either significant symptoms during the placebo challenge only (n= 18) or had no (n=16) or similar (n=5) symptoms during both challenges (unpublished data). Symptoms leading to the suspicion of CMPA and those provoked by the CM challenge are depicted in Table 8. Of the five patients with symptoms during both challenge periods without a clear difference between them, four could tolerate CM in the open challenge, but the parents of one patient chose not to try CM openly (even though the child had relatively minor symptoms during the active challenge period).

The most common symptom reported for the eventually challenge-positive patients was loose stools or diarrhoea, which also recurred in all of them during the active challenge. One additional child, for whom only vomiting had been reported anamnestically, also experienced diarrhoea beginning 24 hours after the start of the active challenge. Vomiting was reported as a presenting symptom in equal frequencies among 17% of the DBCFC-positive patients and 18% of the DBCFC-negative patients. Vomiting within 24 hours of the start of the challenge was reported for three DBPCFC-positive patients, two of whom also experienced persistent feeding difficulties (unpublished data). For the challenge-negative patients, vomiting also typically began within the first 24 hours.

Crying/fussiness was the most frequent placebo symptom, occurring in 30/39 (77%) children with negative DBPCFC. In the challenge-positive group, excessive crying or fussiness only transpired together with either vomiting or loose stools/diarrhoea. There was evidence of concurrent microbial infection (fever, coughing, rhinorrhoea, etc.) in 5/18 DBPCFC-positive children during the active challenge.
<table>
<thead>
<tr>
<th></th>
<th>Original symptoms</th>
<th>Challenge symptoms</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DBPCFC positive,</td>
<td>DBPCFC positive</td>
<td></td>
</tr>
<tr>
<td></td>
<td>n=18</td>
<td>n=18</td>
<td></td>
</tr>
<tr>
<td>Crying/fussiness</td>
<td>11 (56%)</td>
<td>8 (44%)</td>
<td>Ns</td>
</tr>
<tr>
<td>Loose stools</td>
<td>14 (78%)*</td>
<td>15 (83%)#</td>
<td>*p=0.0432</td>
</tr>
<tr>
<td>Vomiting/GER</td>
<td>8 (44%)</td>
<td>3 (17%)</td>
<td>Ns</td>
</tr>
<tr>
<td>Constipation</td>
<td>2 (11%)</td>
<td>0</td>
<td>Ns</td>
</tr>
<tr>
<td>Skin manifestations</td>
<td>5 (28%)</td>
<td>2 (11%)</td>
<td>Ns</td>
</tr>
</tbody>
</table>

Table 8. Symptoms reported by parents as leading to the suspicion of CMPA and during the DBPCFC (modified from (I)).

However, since the gastrointestinal symptoms that recurred during the challenge (as discussed with the parents before opening the code for the blinding) were similar to those reported before the challenge, the DBPCFCs were deemed positive. Concomitant upper respiratory infections were typical overall: suggestive symptoms were reported for five DBPCFC-positive children during the placebo challenge and for 13 patients in the DBPCFC-negative group during either challenge period.

The mothers of the children in both groups were of similar age (see Table 7). Maternal education and the likelihood of the child being firstborn were also somewhat higher among mothers with children in the challenge-negative group, without reaching statistical significance though. Self-reported atopic/allergic disease in the parents was common: the proportion of parents reporting food or pollen allergies, allergic rhinitis or asthma was the same for the two patient groups and the controls. Feeding-related problems (food refusal, vomiting or temporal gagging related to feeding, problematic feeding practices) reported at baseline were significantly high in both groups, reported for 67% of the eventually DBPCFC-positive patients, as opposed to 31% of the DBPCFC-negative patients (post-hoc analysis). These symptoms tended to be resolved more easily for the DBPCFC-negative patients, whereas every third DBPCFC-positive patient still reported feeding difficulties at the six-month follow-up (unpublished data).
5.2 Laboratory parameters (I-II)

5.2.1 General laboratory measurements

Peripheral blood eosinophilia (values above 0.4 E9/l) occurred in all three groups with similarly frequency (see Table 9). Only one of the two DBPCFC-positive patients (presenting with loose stools) who exhibited raised baseline eosinophil counts had slightly higher levels after the CM challenge (pre-challenge 7%, post-CM challenge 10%). There were no significant differences between the patient groups either before or after the challenge or between the control patients regarding peripheral blood haemoglobin, total leukocyte, neutrophil or lymphocyte counts (unpublished data).

The (geometric) means of high-sensitivity CRP levels tended to be slightly higher in the DBPCFC-positive group than in the challenge-negative group after provocation with CM (unpublished data). If the CRP values above 5 mg/l (indicating possible concurrent microbial infection in those two patients) are discarded, the difference was further diminished (0.35 mg/l vs. 0.25 mg/l, p>0.1). There was no correlation between the CRP levels and faecal markers of inflammation.

The patients’ stool samples were tested for occult blood while they were on the CM elimination diet (i.e. before the DBPCFC) and after both challenge cycles; none of the DBPCFC-positive patients tested positive for stool haemoglobin after the CM challenge.

5.2.2 Skin prick testing and cow’s milk-specific IgE

The skin prick tests for CM were positive in two patients with positive DBPCFC (3 mm and 5 mm, the latter had occasional skin rashes and urticaria) and in two patients with negative DBPCFC and without a history of skin manifestations (3 and 4 mm). Other observed positive skin prick test results included the reactions of several DBPCFC-negative patients to hen’s egg (6, 3 and 6 mm), barley (3 mm) and rye (5 mm). One DBPCFC-positive child had a 5 mm skin prick test for wheat (with
suspicion of wheat-induced GI symptoms only). As to the CM-specific IgE, one control patient had a level of 1.01 kU/l without suspicion of CMPA, whereas the CM-specific IgE levels were negative (below 0.35 kU/l) for all study patients.

<table>
<thead>
<tr>
<th>Diet</th>
<th>Fecal Calprotectin, geometric mean (95% CI)</th>
<th>DBPCFC negative n=39</th>
<th>DBPCFC positive n=18</th>
<th>p-value</th>
<th>Control patients n=21</th>
</tr>
</thead>
<tbody>
<tr>
<td>CM free</td>
<td>28 µg/g (21-36)</td>
<td>53 µg/g (33-86)</td>
<td>0.0203</td>
<td>25 µg/g (13-50)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Lactase genotype</th>
<th>NA</th>
<th>11/35 (31%)</th>
<th>4/17 (24%)</th>
<th>0.7465</th>
<th>4/21 (22%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC13910, n (%)</td>
<td>NA</td>
<td>11/35 (31%)</td>
<td>4/17 (24%)</td>
<td>0.7465</td>
<td>4/21 (22%)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fecal Hemoglobin, no. positive</th>
<th>CM free</th>
<th>3</th>
<th>2</th>
<th>NS</th>
</tr>
</thead>
<tbody>
<tr>
<td>CM+</td>
<td>1</td>
<td>0</td>
<td>NS</td>
<td></td>
</tr>
</tbody>
</table>

| Cow’s milk specific IgE, no. >0.35 kU/l | NA | 0 | 0 | NS |

<table>
<thead>
<tr>
<th>B-Eosinophils &gt; 0.4 E9/l</th>
<th>CM free</th>
<th>5</th>
<th>1</th>
<th>NS</th>
</tr>
</thead>
<tbody>
<tr>
<td>CM+</td>
<td>7</td>
<td>2</td>
<td>NS</td>
<td></td>
</tr>
</tbody>
</table>

**Table 9.** Laboratory parameters for the study patients and controls.

5.2.3 The Lactase CT<sub>13910</sub> genotype

The lactase CT<sub>13910</sub> genotype associates with adult-type hypolactasia, while individuals carrying the CC genotype are at risk of hypolactasia symptoms after childhood. We examined this genotype in 52 out of the 57 patients. For the five remaining patients, we encountered technical problems with the sampling (not enough blood drawn or errors in test processing). In the DBPCFC-negative patients, we detected the CC<sub>13910</sub> genotype in 11/35 (31%) patients. The prevalence of the CC<sub>13910</sub> genotype in Finland is 18% [Enattah 2002], so there was a difference between the observed proportion of CC<sub>13910</sub> genotypes in the DBPCFC-negative patients in our cohort and their prevalence in the overall population (p=0.0387).
5.2.4 Stool markers of inflammation

The DBPCFC-positive patients had significantly higher levels of faecal calprotectin (also with quite high inter-individual variability) manifesting already before the challenges, when they were on a CM-free diet (Figure 4). This difference between the patient groups was evident during both the placebo and CM challenges, even though the statistical significance was lost due to the high degree of variability. The within-group variance was especially high for the DBPCFC-positive patients. The numbers of patients exhibiting values above the generally accepted reference of 100 μg/g are listed in Table 10. The chances that a positive predictive value for faecal calprotectin greater than 100 μg/g correctly indicates DBPCFC positivity is only 0.44, while the chances are even lower for a negative predictive value: 0.21.

Faecal β-defensin 2 (F-BDEF2) levels tended to be higher (without reaching statistical significance) in the DBPCFC-negative patients (Figure 5), both when they were following a CM-free diet and after provocation with CM. There were no significant differences between the faecal IgA levels in the different patient groups.

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>After CM provocation</th>
<th>After placebo provocation</th>
</tr>
</thead>
<tbody>
<tr>
<td>DBPCFC-positive patients, n=18</td>
<td>5 (28%)</td>
<td>6 (33%)</td>
<td>4 (22%)</td>
</tr>
<tr>
<td>DBPCFC-negative patients, n=39</td>
<td>4 (10%)</td>
<td>1 (3%)</td>
<td>7 (18%)</td>
</tr>
<tr>
<td>Control patients, n=21</td>
<td>2 (10%)</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

Table 10 Numbers of patients with faecal calprotectin values above 100 μg/g at baseline (CM-free diet for the study patients and unrestricted diet for the control patients), and after provocations with CM and placebo ($\chi^2$ test for this table: p=0.0027).
Figure 4 Faecal calprotectin levels in young children undergoing the DBPCFC for cow’s milk and control patients with no diet restrictions or gastrointestinal symptoms. The p-value is <0.05 (ANOVA) for comparing the controls and the two patient groups, both before the challenges and after the CM challenge. The graph shows whiskers at the 10–90th percentiles with a line at the medians.
Figure 5. Levels of faecal β-defensin 2 (A) and faecal IgA (B) in children suspected of GI-CMPA. None of the differences were statistically significant. The graph shows the median and Tukey’s whiskers.

5.2.5 Cow’s milk-specific IgG, IgG subtypes and IgA (II)

We measured the levels of the serum antibodies against CM α-casein and β-lactoglobulin as well as for ovalbumin (hen’s egg antigen). The results for the total IgG, IgG1 and IgG4, as well as the total IgA are depicted in Figure 6. There were no differences between the three groups (the DBPCFC-positive group, the DBPCFC-negative group and the control patients) regarding concentrations of antibodies against ovalbumin, with all groups displaying similarly low levels (see Figures 6A and 6B).
The levels of IgG4 against α-casein and β-lactoglobulin as well as the total α-casein IgG were generally low in the study patients, with significant difference compared to the controls (p<0.01), but without differences between the DBPCFC-positive patients and the DBPCFC-negative patients. The levels for β-lactoglobulin, total IgG and IgG1, and α-casein IgG1 were similar between the three groups. In terms of infants one year of age or older, 18/21 (86%) in the patient groups (regardless of their DBPCFC status) and 6/13 (46%) in the control group had undetectable α-casein IgG4 (p=0.0223).

**Figure 6A.** Levels of serum IgG, IgG1 for cow’s milk α-casein and β-lactoglobulin and for ovalbumin in patients following a cow’s milk-free diet due to gastrointestinal symptoms suspected of cow’s milk allergy, stratified by their cow’s milk challenge results (DBPCFC-positive and DBPCFC-negative patients), as well as in controls without diet restrictions or food allergy symptoms. The p-values were first calculated by comparing the three groups using the Kruskall-Wallis statistic (ANOVA); if p<0.05, then further p-values between the two groups were calculated using the Mann-Whitney test.
**Figure 6B.** Levels of serum IgG4, IgA for cow’s milk α-casein and β-lactoglobulin and for ovalbumin in patients following a cow’s milk-free diet due to gastrointestinal symptoms suspected of cow’s milk allergy, stratified by their cow’s milk challenge results (DBPCFC-positive and DBPCFC-negative patients), as well as in controls without diet restrictions or food allergy symptoms. The p-values were first calculated by comparing the three groups using the Kruskall-Wallis statistic (ANOVA); if p<0.05, then further p-values between the two groups were calculated using the Mann-Whitney test.
5.3 Child Behavioural Characteristics (III)

5.3.1 The Parenting Stress Index Child Domain

The total scores for the PSI Child Domain were high in patients with suspicion of GI-CMPA (Table 11). The differences in the median scores between the two patient groups were not significant either at baseline (p=0.2937) or at the follow-up (p=0.2257), whereas the medians were significantly different (p<0.001) for all patients suspected of GI-CMPA compared to the controls. The scores were significantly lower after six months in both patient groups, although the drop-out rate was considerable.

<table>
<thead>
<tr>
<th>All patients</th>
<th>Controls</th>
<th>p-values (all vs. ctrl)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PSI Child Domain scores at baseline</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of returned questionnaires</td>
<td>48 (84%)</td>
<td>17(80%)</td>
</tr>
<tr>
<td>Total score, median (range)</td>
<td>114 (71–169)</td>
<td>84 (66–137)</td>
</tr>
<tr>
<td>No. of patients above 90th percentile#</td>
<td>11 (23%)*</td>
<td>1</td>
</tr>
<tr>
<td>Demandingness, median (range)</td>
<td>22 (10–37)</td>
<td>18 (12–30)</td>
</tr>
<tr>
<td><strong>PSI Child Domain scores at 6-month follow-up</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of returned questionnaires</td>
<td>36 (63%)</td>
<td></td>
</tr>
<tr>
<td>Total score, median (range)</td>
<td>91 (61–146)</td>
<td></td>
</tr>
<tr>
<td>p-value (baseline/follow-up)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 11. Results of the Parenting Stress Index (PSI) Child Domain scores at baseline and at the six-month follow-up (*p-value against 10% expected proportion; # 90th percentile of the normative values) [Abidin 1995].
5.3.2 The Infant Temperament Questionnaire (ITQ)

Regarding the ITQ, the subscale of Difficultness was also significantly affected in the patients, regardless of their DBPCFC status (see Table 12). The median scores for the DBPCFC-negative patients (21 points [7-35]) and the DBPCFC-positive patients (18 points [12-30]) were similar (p=0.6336), but they were significantly higher (ANOVA p=0.0045) than for the control patients. After six months, the median scores in both patient groups had slightly decreased and no longer differed from those of the controls (ANOVA p= 0.1062).

<table>
<thead>
<tr>
<th>ITQ Difficultness scores at baseline</th>
<th>All patients (n=49)*</th>
<th>Controls (n=17)*</th>
<th>p-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of returned questionnaires</td>
<td>44 (90%)</td>
<td>12 (70%)</td>
<td>ns</td>
</tr>
<tr>
<td>Difficultness, median (range)</td>
<td>20 (7–35)</td>
<td>12.5 (7–23)</td>
<td>0.0008</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>ITQ Difficultness scores at 6-month follow-up</th>
<th>All patients (n=49)*</th>
<th>Controls</th>
<th>p-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of returned questionnaires</td>
<td>31 (63%)</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Difficultness, median (range)</td>
<td>16 (3–31)</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>p-value (baseline/follow-up)</td>
<td>0.0212</td>
<td>NA</td>
<td></td>
</tr>
</tbody>
</table>

Table 12. The Infant Temperament Questionnaire (ITQ) Difficultness subscale scores in patients with suspicion of CMPA and controls.

* this questionnaire was only used in infants below 18 months of age.
5.4 The Emotional Availability Scales (IV)

The EAS scores had significantly different distributions in the study patients compared to the normative data. Although the difference in the total scores between the DBPCFC-positive patients (n=7) and DBPCFC-negative patients (n=17) did not reach statistical significance, compared to the normative data the DBPCFC-negative patients had lower scores significantly more often. The observed EA profile is depicted in Table 13. The median total score for all patients (n=24) combined was 30 points (range 21–39), whereas the normative [Salo 2010] total (n=35) was 32 (28–36), p=0.0380.

<table>
<thead>
<tr>
<th>EAS scales</th>
<th>DBPCFC negative (n=17)</th>
<th>DBPCFC positive (n=7)</th>
<th>p-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sensitivity</td>
<td>4.5 (3.5–7)</td>
<td>6 (4–6)</td>
<td>0.0175</td>
</tr>
<tr>
<td>Structuring</td>
<td>5 (4–6)</td>
<td>6 (3–7)</td>
<td>0.5028</td>
</tr>
<tr>
<td>Nonhostility</td>
<td>5 (4–7)</td>
<td>7 (5–7)</td>
<td>0.02754</td>
</tr>
<tr>
<td>Intrusiveness</td>
<td>5 (3–7)</td>
<td>6 (4–7)</td>
<td>0.0349</td>
</tr>
<tr>
<td>Child</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Responsiveness</td>
<td>5 (3–6)</td>
<td>5 (3–7)</td>
<td>0.0265</td>
</tr>
<tr>
<td>Involvement of mother</td>
<td>4 (2–6)</td>
<td>4 (3–6)</td>
<td>0.0390</td>
</tr>
</tbody>
</table>

Table 13. The results for the Emotional Availability Scales showing the Kruskall-Wallis test p-values (comparing the DBPCFC-positive, DBPCFC-negative and normative groups).
This thesis demonstrates that suspicions of CMPA based on parental perceptions of a child’s gastrointestinal symptoms may in fact be unfounded in every two out of three patients. According to the presented data, this is likely due to the multifactorial origin of the gastrointestinal symptoms in early childhood, with several other feeding-related causative factors confounding the observations. This study includes the largest published prospective cohort focusing on the gastrointestinal manifestations of CMPA thus far, with the diagnosis based on the gold standard of FA diagnostics: the DBPCFC. Study I streamlined the symptomology associated with GI-CMPA by demonstrating that the only symptom positively associated with CMPA was diarrhoea/loose stools. This study also highlighted the important relationship between feeding problems and GI-CMPA symptoms. This thesis, sub-study II, reported the levels of several immunological markers associated either with GI symptoms or CMPA; none of these markers was proven to aid in the diagnostic process, though. The impaired development of IgG4 class antibodies to CM-specific proteins in patients on a CM-free diet compared to the controls who consumed CM normally was concerning. This may have an impact on the process of gaining tolerance to CM, and it further underlines the need for an accurate diagnosis of GI-CMPA. Studies III-IV report for the first time in medical literature on the frequently problematic mother–child emotional interactions and maternal perceptions of a child’s characteristics in patients suspected of GI-CMPA.
6.1 Clinical aspects of gastrointestinal manifesting cow’s milk protein allergy

Young infants and children suspected of CMPA because of gastrointestinal symptoms were recruited to participate in the present study. Fifty-seven patients completed the study protocol for the DBPCFC; one-third of the patients exhibited significant symptoms during the CM provocation (deemed DBPCFC-positive patients). This proportion is in line with previous studies [Baehler 1996; Dambacher 2013; Gellerstedt 2004; Hospers 2006]. For another third of the patients, the symptoms reported during the placebo provocation were so remarkable that parents falsely identified this provocation as containing CMP. This proportion of placebo reactions is so remarkable that the use of an open food challenge in GI-CMPA should be regarded with caution. The young age of the patients when the symptoms first started (median 3 months) and at diagnosis (median 8.7 months), with a median 2.5 month duration of a CMP-free diet before diagnosis, are typical in our tertiary care hospital for infants suspected of GI-CMPA. The rather long median duration of the cow’s milk elimination period is mostly due to parentally imposed elimination diets, and many of the infants had already been tested for cow’s milk at home during this period, with perceived intolerance. Tolerance to CMP in CMPA patients has been shown to develop after one year of age [Saarinen 2005], so it is unlikely that a developed tolerance would explain the small proportion of challenge-positive patients. The symptoms described by the parents were significant and so disruptive that the parents also felt it prudent to perform the rigorous DBPCFC protocol.

The lack of patients with FPIES-like symptoms in the study cohort is acknowledged, but not surprising because this most severe form of GI-CMPA is rare. Less severe symptoms, such as irritability and GERD-like symptoms, dominate the present-day GI-CMPA suspicions in the Greater Helsinki area. This study only included patients with remarkable and troublesome symptoms lasting for several weeks. Only the parentally reported diarrhoea/loose stools as a presenting symptom was associated with eventual CMPA diagnosis. However, this symptom did occur in the DBPCFC-negative patients during the placebo provocation as well, and thus the need for a DBPCFC cannot be waived. Vomiting/GERT-like symptoms developed at a similar level of frequency during the CM provocation in the DBPCFC-positive patients and during the placebo provocation in the DBPCFC-negative patients. Therefore, vomiting or reflux symptoms as a sole presentation of GI-CMPA should be regarded with caution. It has been proposed that constipation associates with CMPA. However, constipation related to CMPA was not noted in this IgE-negative cohort while undergoing a five-day CM challenge. In the medical literature on infant hematochezia, the evidence indicating an association with CMPA seems to be relatively infrequent [Arvola 2006; Xanthakos 2005]. None of the DBPCFC-positive children in the present study tested positive for stool blood. Patients with minor skin
manifestations were not excluded and skin rashes were reported during the challenges only infrequently, with no association with the CMP provocation of GI symptoms. Non-IgE-mediated skin manifestations of CMPA are indeed rare. In one study, only one-third of the patients (mean age 2.5 years) with atopic eczema/dermatitis had FA [Eigenmann 1998].

The clinical laboratory parameters showed that none of the patients suspected of GI-CMPA tested positive for cow’s milk-specific IgE. Two patients in both the DBPCFC-positive and DBPCFC-negative groups had slight SPT reactions to CM, but these reactions were only within 50% of the diagnostic probability range [Verstege 2005], which coincided with the DBPCFC results. The use of cow’s milk-specific total IgG or IgA measurements is useless for diagnosing GI-CMPA, a conclusion supported by the present study. In population-based studies, a high degree of variability in cow’s milk-specific IgG has also been noted in asymptomatic individuals [Hochwallner 2011; Oldaeus 1999; Zeng 2013]. The highest values for both CM-IgG and IgA presented here were in fact noted among the control group patients. The frequency of levels higher than the reference level was similar in the DBPCFC-negative and DBPCFC-positive patients. The same applies to the levels of α-casein and β-lactoglobulin IgG, IgG4 and IgA: higher values were detected in the control group using CM. No significant differences emerged between the DBPCFC-positive or DBPCFC-negative patients.

The frequency of patients exhibiting zero levels of α-casein and β-lactoglobulin IgG4 antibodies was high. Compared to the control group, the differences were significant, but there was no distinction between the two study groups. The major differentiating factor between the three groups was the CMP-free diet of the patients. Confirming evidence is available: the use of CM at an early age associates with later higher levels of α-casein and β-lactoglobulin IgG4 [Jenmalm 1998; Savilahti 2010b]. An association of low levels of α-casein IgG4 with FPIES (n=11) has also been reported [Shek 2005]. The FPIES patients in that particular study had a median age of 40 months. Considering the generally accepted diagnostic and management criteria associated with FPIES, a prolonged CM-free diet may well have influenced the results in that study. Since IgG4 is implicated in tolerance to CMP, the low levels of α-casein (and β-lactoglobulin) IgG4 in patients on a CM-free diet could mean that the elimination diet interferes with the development of tolerance [Järvinen 2013]. This finding is an important factor when considering the management of symptoms suspected of GI-CMPA (Figure 7). Evidence regarding the tolerogenic effect of CM-derived peptides is just emerging [Berni Canani 2013a; Meulenbroek 2013], indicating the benefit of actively reintroducing these CM fragments to a child's diet.
Regarding the adult-type hypolactasia genotyping, there was a small but significant difference between the two patient groups, with the DBPCFC-negative patients exhibiting the CC\textsubscript{1390} genotype (which associates with later childhood/early adulthood hypolactasia) in excess of the expected frequency. The lactase enzyme coding gene in itself remains intact, while the polymorphism located in a distant region regulating the lactase gene expression (promoter region) mutates. In adults, this CC\textsubscript{1390} allele also associates with unexplained abdominal pain when controlled for cow’s milk consumption [Anthoni 2007]. In the majority of Caucasian children, the lactase enzyme activity only starts to decline after preschool age [Wong 2007]. However, colicky infants have been found to exhibit abnormal breath hydrogen testing following lactose ingestion, which is suggestive of carbohydrate malabsorption [Rhoads 2009]. In young rats, the dietary carbohydrate and fat contents regulate the jejunal lactase enzyme activity [Tanaka 2008], but the mechanisms for regulating lactase enzyme activity in human children are not completely understood [Järvelä 2009]. Low-lactose formulas (with intact CMP) have shown controversial benefits in colicky infants [Lucassen 2010].
Faecal calprotectin levels in the study patients showed high variability, but significantly higher values were found in the DBPCFC-positive patients (n=18) both after CMP provocation and during CMP elimination. The concentration means in the DBPCFC-positive patients were slightly above the 50 μg/g cut-off value proposed by Fagerberg et al. [2005]. However, this test cannot be used to diagnose GI-CMPA due to the widespread distribution of calprotectin concentrations. Calprotectin is derived predominantly from neutrophils, with monocytes and reactive macrophages being the other sources. Calprotectin levels in stool correlate with the presence of neutrophils in the gut mucosa [Gisbert 2009]. At the age of three months, infants on CM formula as opposed to breast milk had lower calprotectin levels [Savino 2010], thus negating the assumption that CMP as such would cause elevated levels. There is a high degree of variation in the stool calprotectin levels of healthy term infants overall [Kapel 2010]. The intestinal colonisation of especially clostridia and staphylococci increases the mucosal calprotectin excretion in newborns [Rouge 2010]. Low-grade mucosal inflammation may enhance the development of allergies in susceptible patients by increasing gut mucosal permeability [Perrier 2011]. Faecal ECP and IgA expressions were also higher in healthy breast-fed infants at four months, but by nine months of age they had decreased significantly [Amarri 2006], indicating a maturing intestinal barrier function.

The elevated calprotectin levels observed as a part of this study suggest an ongoing low-grade gut mucosal inflammation, which also persisted during the CMP-free diet in the challenge-positive children. This finding certainly needs to be further studied in non-IgE CMPA. Research has failed to demonstrate a uniform CMP-specific immunological reaction behind the GI-CMPA. Could it be that the mucosal reaction to CMP is in fact not allergen specific? The existing literature and clinical experience suggest that some CMPA patients experience non-specific reactiveness to solid foods the first time they are introduced [Beauchamp 2011; Nowak-Wegrzyn 2003]. Such a presentation is not typical of allergic reactions.

Further evidence suggesting the involvement of a gut microbiota–mucosal immunity axis in the pathogenesis of symptoms associated with GI-CMPA comes from the faecal β-defensin 2 levels. Even though the present study failed to show consistently significant changes, there was a trend towards higher values in the DBPCFC-positive patients, both during the CM-free diet and after CM provocation. β-defensins are expressed in the intestinal mucosal epithelial cells, with increasing expression depending on gestational age. They function as part of the innate immunity as antimicrobial agents, with the ability to activate antigen-presenting cells [Richter 2010]. The expression of β-defensin 2 is associated with bacterial colonisation, with higher values found in patients with ulcerative colitis (but less so with Crohn’s disease) and slightly elevated values in adult IBS patients [Langhorst 2009]. Higher levels of β-defensin 2 at six months of age are associated with a later risk of atopic
sensitisation [Savilahti 2012]. Research into the gut microbiota in allergic diseases is ongoing, with evidence of risk reduction when using certain probiotics [Elazab 2013].

The choice of a CMP-free formula with suspicion of GI-CMPA deserves attention. In the present study, the use of AAF and SF was surprisingly frequent (in one-third of patients) when considering the research-based frequency of allergy/intolerance to EHF, which is 2% [Klemola 2002]. It is likely that many children have first tried EHF without exhibiting any symptoms of GI-CMPA, but AAF has been the first choice for some children because of perceived severe symptoms or sibling history. Amino acids cause an osmotic load in the lumen of the gut, resulting in softer stools. They are also biochemically active molecules that, for example, cause the colour of the stools to become dark green. The only AAF (and SF) available in Finland is lactose free, which further adds to the confounding variables associated with the perceived elimination responses of the children under study. The use of AAF or SF was not associated with an increased likelihood of GI-CMPA in this study, and intolerance to EHF was not detected in any of the patients. It may take children a longer time to gain a tolerance to CMPA when using AAF and SF [Berni Canani 2013b]. There is evidence suggesting that maternal use of CMP through pregnancy and lactation reduces the risk of CMPA [Tuokkola 2011]. The small number of CMP-derived peptides in EHF may be tolerogenic [Meulenbroek 2013]. AAF should only be used for GI-CMPA if intolerance to EHF has been demonstrated (preferably via a DBPCFC).

The protocol for the DBPCFC employed in this study was feasible and safe. International expert advice on how to perform the DBPCFC focuses, however, on finding the smallest eliciting doses and achieving nearly 100% diagnostic certainty [Sampson 2012]. Such protocols are time consuming because they recommend several repeated challenges per protocol and increasing the dosages; this makes the challenge impractical for use in clinical practice. The protocol applied here (I) was simple and straightforward, but it cannot rule out a number of false-positive results. Repeating the challenges would have reduced their number, but this was not feasible in light of the rate of feeding-related problems. The number of patients who discontinued the DBPCFC was low, with the most common reasons being bottle refusal and inadvertent CM ingestion at home without exhibiting any symptoms. The symptoms exhibited during the first challenge cycle caused one patient to be withdrawn from the study. In most cases, the reasons to withdraw from the study were multifactorial, but the parents were not required to state the reasons for withdrawing in detail. However, since most of the patients who withdrew from the study had concluded the DBPCFC in the regular outpatient clinic setting, opposition to the DBPCFC is unlikely. Severe reactions did not occur, with the only immediate reaction (within two hours of digestion) being vomiting in one child who later proved to be CMPA negative (but with pronounced feeding-related symptomology).
The prognosis for non-IgE CMPA is favourable. With respect to FPIES, nearly 100% of children tolerated CMP at two years of age [Hwang 2009], whereas other research has shown that a tolerance to non-IgE CMPA is achieved by three years of age [Saarinen 2005]. In terms of the present study, one-third of the DBPCFC-positive patients could tolerate small amounts (often cooked/processed) of CMP already after six months. Since no severe immediate reactions were observed in the DBPCFC-positive patients, a recommendation was given to try CMP at home every three months. However, most parents did not do so, indicating a fear of recurring symptoms as the reason. In light of the mild/modest symptoms during the significant CMP provocation with the DBPCFC, this parental apprehension should be taken into account when planning the follow-up for GI-CMPA patients [Strinnholm 2010].

6.2 Psychological considerations related to the suspicion of cow’s milk protein allergy with gastrointestinal symptoms

This study has demonstrated that the unspecific symptoms in young children and infants suspected of GI-CMPA often occur together with certain psychological aspects of the child and the caregiver. The connection between feeding-related problems and symptoms and GI-CMPA needs to be discussed. Parental reports on the symptoms of children suspected of GI-CMPA are temporarily connected to feeding the child. Traditionally, physicians have focused on the contents of the feeding bottle (CMP, lactose, other ingredients in infant formula) as causing the child’s symptoms. This thesis introduces a new facet to this assessment by proposing the feeding situation and interaction between the child and mother as yet another cause of the parentally perceived symptoms. Furthermore, this study has shown that a significant portion of the infants suspected of GI-CMPA are perceived as temperamentally difficult by their mothers. The questions incorporated in the studied temperament measures do not address gastrointestinal problems, but focus instead on the more general aspects of infant life, such as reactions to new stimuli in the child’s environment, biological regularity (e.g. cycle of the day) and demandingness of parental attention. An infant’s temperament has previously been associated with parental perceptions (often without objective data confirming the increased problem behaviour) of excessive crying and fussing, sleeping problems and feeding practices [Barr 1989; Hayes 2011; Lehtonen 1994; Mcmeekin 2013]. Figure 8 depicts the possible inter-relations of feeding-associated symptoms with mother–child emotional interaction and parental perceptions of a child’s temperament characteristics.
Figure 8. Suggested etiology of feeding-related problems in children suspected of GI-CMPA, as proposed by the author.

An important aspect to consider is that the questionnaires only depict maternal perceptions of a child’s behavioural characteristics. Here, maternal psychology also plays an important role, as prepartum maternal anxiety has been closely related to infant temperament outcomes [Vaughn 1987]. Thus, the parental perceptions of a child’s symptoms should be interpreted with caution and possible parental anxiety should be addressed as well.

The level of emotional availability in mother–child interactions has been studied in infant feeding disorders. The pattern of problematic interactions studied using the EAS in the present study resembled that found in infant FD [Gueron-Sela 2011]. The associated problems pertain to relative maternal insensitivity to a child’s signs and cues and a tendency for intrusiveness (pushing herself and/or her ideas onto the child). The child in this interactive environment shows relative unresponsiveness to her or his mother’s attempts at interaction and the extent to which the mother impedes the interaction. This pattern of interaction may represent an interactive approach where the mother is pushing too hard to involve the child with insufficient positive affective scaffolding and support. This, in turn, may cause the child to actually become less responsive, as we observed. The problematic emotional interaction profile occurred significantly more frequently in those patients with
negative DBPCFC rather than positive DBPCFC, indicating that there may be an organic basis to the reported feeding problems of DBPCFC-negative patients with CMPA more often than for DBPCFC-positive patients.

Regardless of the origins of the discovered emotional interaction patterns or parental perceptions of a child’s characteristics, the presented observations may have an impact on the child’s well-being in the future. Studies with larger groups of patients are clearly needed to confirm our results.
6.3 Suggestions for clinical management of patients with suspected GI-CMPA

First, the symptoms leading to the suspicion of GI-CMPA in infants and young children below the age of four must be discussed in detail with the parents. Excessive crying or fussing in the absence of diarrhoea, objective signs of disease in the child or other GI symptoms are unlikely to be caused by CMPA and formula changes should be discouraged (Figure 9). Specific questions about the feeding and general mother–child emotional interactive environment may help in deducing the extent of psychological maladjustment. Undue parental anxiety should likewise be addressed and the need for psychological counselling considered. If the symptoms and history are strongly suggestive of GI-CMPA, a CMP-free diet may be prescribed. In lactating mothers, the need for total CM elimination may be unnecessary because the amounts of bovine β-lactoglobulin in breast milk are quite low (0-100 μg/l, corresponding to approximately 1:10 000 in the contents of regular CM formula protein) [Jakobsson 1991]. The first choice of formula is EHF, as discussed above. A positive response to CMP elimination should be followed by a DBPCFC. If there is no response, or only a partial response, to the elimination diet, the likelihood of CMPA is low and this should be addressed and other functional childhood GI disorders considered [Hyman 2006]. If either AAF or SF produces a positive elimination response, then the need for AAF must be checked regularly. The large number of placebo symptoms should best be discussed before the DBPCFC to reduce parental anxiety after the challenge. In the absence of severe and immediate symptoms, re-challenges for CMP can be performed at home with small amounts of (possibly cooked or processed) CM. Parental anxiety and reluctance to perform these re-challenges may result in prolonged unnecessary elimination diets, which may be detrimental to the child [Isolauri 1998]. The follow-up plan should be individualised to take that aspect into account.
Figure 9. Algorithm for managing infants and young children with GI symptoms suspected of CMPA.
6.4 Strengths and limitations of the study

The major strengths of this study include its prospective recruitment and follow-up as well as the clinical settings. The children included in the study were recruited based on referrals to the outpatient clinic in a tertiary care hospital for strong suspicion of GI-CMPA, which was based in most cases on repeated open CM challenges by the parents or the referring physician. A selection bias towards clinically mild symptoms or disease is unlikely since the patients had a long history of being on a CM-free diet and the fact that the parents also agreed to the rather demanding DBPCFC protocol. Clinical suspicion of GI-CMPA has evolved over the years to resemble that described in the present study, with a minority of patients experiencing the severe symptoms of FPIES. However, since the study patients represent infants and young children below the age of four with rather severe symptoms and a strong suspicion of GI-CMPA, the results must be interpreted with caution in patient populations with different ages or in primary care settings.

Patients were only recruited on a prospective basis, and the DBPCFCs were supervised by the same team (one paediatrician and nurse), which provided uniformity and validity to the results. This study, to the best of our knowledge, is the largest published, single-centre cohort on GI manifestations of CMPA using the DBPCFC to confirm the diagnosis. The use of a blinded, structured analysis tool (the EAS) to describe the emotional interaction between the mother and child significantly adds to the credibility of the findings regarding the psychological associations with GI-CMPA.

There are some limitations pertaining to the present study, however. The number of study patients was estimated on the basis of 1:1 DBPCFC-positive versus DBPCFC-negative patients. The number of positive DBPCFC reactions was smaller than expected, meaning that the power to detect possible minor differences between the two DBPCFC outcome groups is low. This affects especially the EAS sub-study. The rate of positive challenges should be regarded with caution. There are likely to be several false positives, as suggested by the high frequency of observed placebo reactions. Performing the DBPCFC twice would have made the results more robust, but this was considered unethical since nearly 80% of the DBPCFC-positive children showed signs of clinically significant feeding problems. The DBPCFC protocol used was aimed at discovering symptoms occurring within 72 hours, suggesting a hypersensitivity disorder as the causal mechanism. Other CM-associated causative factors, such as lactose malabsorption, functional (including protein content-related) constipation, or the microbiota changes orchestrated by increasing the amounts of CM, may have been overlooked. However, the nearly 100% rate of CM consumption...
in the DBPCFC-negative patients at the six-month follow-up suggests that this was not relevant.

The control group consisted of infants and young children visiting the Skin and Allergy Hospital for reasons other than food allergies or some other atopic disease. As they were not recruited from a healthy population, the results must be interpreted accordingly. The stool samples were transferred to the laboratory at room temperature, and then immediately frozen. The effect of these processes on the F-IgA and F-β-defensin results is unknown, though this practice is acceptable in faecal calprotectin analysis.

With regard to the EA assessment, a longer (up to one hour) video-recording session could have given us more insight into the nature of EA [Biringen 2005] regarding GI-CMPA suspicion. Previous studies using observations lasting 10–15 min have shown the validity of the choice of a 15–20 minute duration for measuring EA [Easterbrooks 2000; Wiefel 2005]. We performed the video recording at the outpatient clinic premises, and with the median patient age being 10 months for the EAS sub-study, longer sessions would have been impractical. Video recording the feeding sessions might have resulted in even lower EAS scores. However, the reproducibility and uniformity of data impacted the video session arrangement even more. The age in the control group was slightly higher than for the patients. While this 4.5 month difference in the median ages may have had an impact on some results, the effect was negligible for most of the results. For instance, the PSI manual only reports reference values beginning at one year of age and for subsequent full years [Abidin 1995].
Cow's milk allergy presenting with gastrointestinal symptoms rarely causes the GI ailments commonly observed in infancy and early childhood. This study shows that in a tertiary care setting, only one in every three suspicions is in fact corroborated by the DBPCFC, the gold standard of FA diagnostics. The DBPCFC protocol used was feasible in clinical practice and also safe. The only symptom associating with a positive DBPCFC was diarrhoea, with vomiting/GER exhibiting similar frequencies in the challenge-negative and challenge-positive patients. Feeding-related problems were common in both groups. Specific laboratory tests that could aid in the diagnostic process could not be identified. In the challenge-positive children, there is a possibility of low-grade gut mucosal inflammatory reaction unresponsive to CMP elimination. The low levels of CM-specific IgG and IgA antibodies in children with a CM-free diet confirm the fact that with GI-CMPA, measuring the CM-specific IgE, IgA or IgG levels is not diagnostic. It also raises a concern about unnecessarily prolonging CMP-free diets, which may slow down the child’s ability to gain a tolerance to CMP. This study also found that the mother–child emotional interaction environment and maternal perceptions of a child’s difficult temperament were frequently affected in children suspected of GI-CMPA.

In conclusion, this study demonstrated that most suspicions of GI-CMPA cannot be ascertained, and that while the exact pathophysiology of GI-CMPA symptoms remains unknown, there is an increased risk of psychological maladjustment in patients with suspicion of GI-CMPA.
Implications for future research

In light of the emerging importance of gut commensal microbiota, further research into the relationship between CM and intestinal microbes is needed. Does CM affect the composition of the microbiota, and which components of CM would then be responsible for these changes? Also, regarding the ambiguous gut mucosal histology and endoscopy findings presented in the literature, a prospective DBPCFC-controlled study on endoscopic findings in GI-CMPA (non-IgE) would give valuable information as to the pathomechanisms of and new treatment modalities for GI-CMPA. Also, a controlled study on the effect of a total elimination of CMP versus protocol-based regular feedings of baked or cooked cow’s milk [Kim 2011] in GI-CMPA is warranted. The role of the mother’s psychology in the development of symptoms in a subgroup of GI-CMPA patients should be further studied: parental anxiety traits and other related factors need to be elucidated. Also, what kind of psycho-education would be useful (and feasible in clinical practice) in managing these patients? The level of interactive problems in the present study was seldom such that they would require a psychiatric evaluation, and thus one could postulate that a lighter approach (counselling, written psycho-education material) would suffice in most cases.
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