Pregnancy and Inflammatory Bowel Disease

perspectives from bench and bedside

Janine van der Giessen

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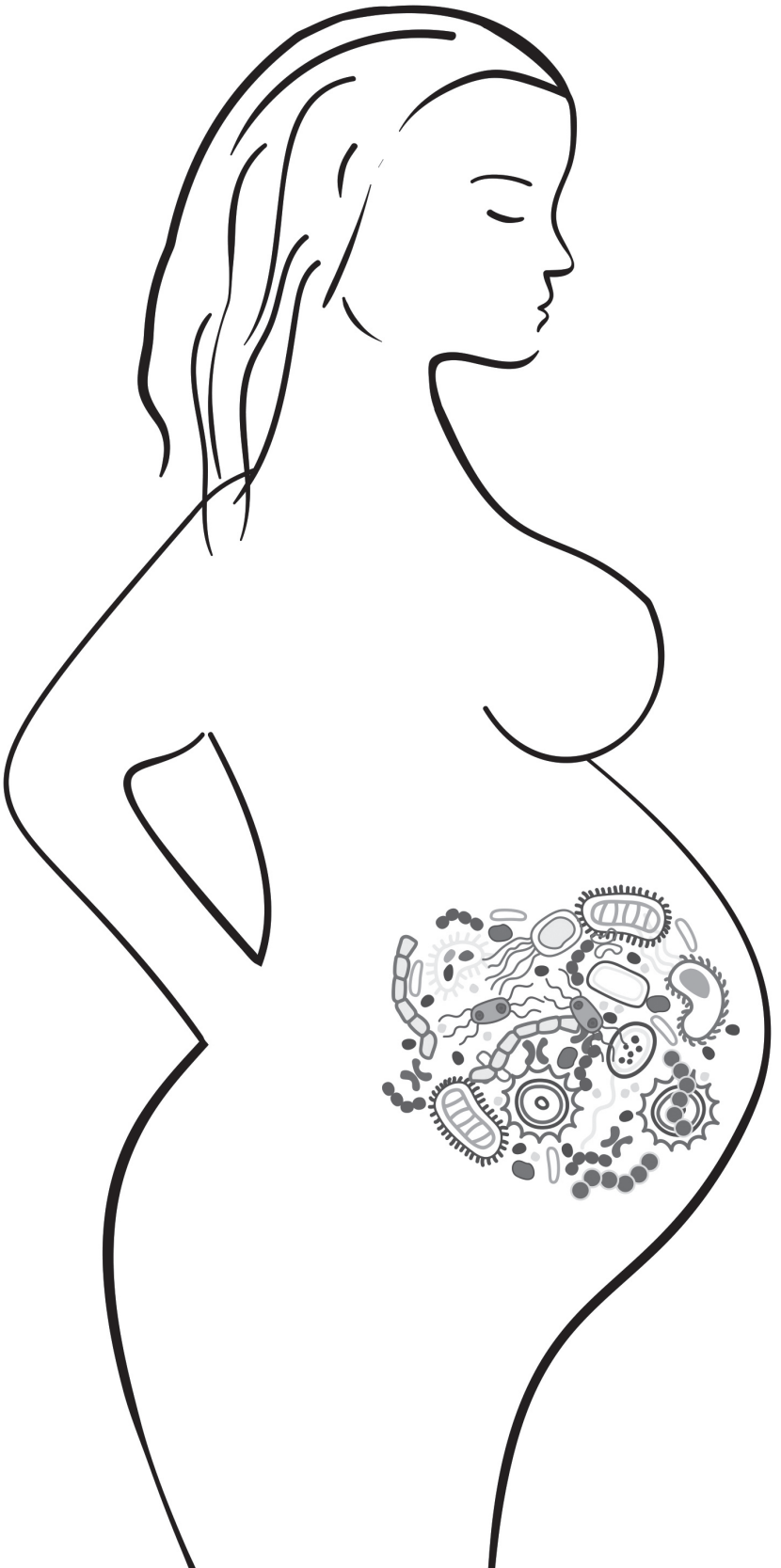

Chapter 1

Introduction and outline of the thesis

Fertility, Pregnancy, and Lactation - *IBD nursing manual 2018*

INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic disease of the gastrointestinal tract that affects men and women in their young and reproductive years of life. Anxieties about potentially harmful medication, the effect of pregnancy on disease, the effect of disease on the fetus and the potential of passing on of disease to offspring result in a relatively high 'voluntary' childlessness in young women with IBD. A careful consultation with the parents-to-be on these justified concerns is necessary and involves a pro-active approach. Since IBD affects men and women in their reproductive years of life, questions arise around fertility and the possible effect thereon of IBD itself or medication. Furthermore, the effect of IBD on pregnancy and the use of medication during pregnancy and lactation should be discussed during preconception counseling.

Fertility in IBD females

Medications prescribed for the treatment of IBD are not associated with lower fertility rates in IBD females as compared to the general population. It is known that if patients are in remission, women are as fertile as the general population (1), however fertility might be reduced in patients with:

- Active Crohn's disease
- Pelvic or abdominal surgery for IBD (e.g. ileal pouch-anal anastomosis (2-4))

Reasons for this decreased fertility probably include induction of inflammation to the ovaries and fallopian tubes in active Crohn's disease and the occurrence of dyspareunia when there is active perianal disease (5). Further, surgical interventions for IBD may cause tubal adhesions. Patients who have an ileal pouch-anal anastomosis (IPAA), or pouch-surgery, have higher rates of tubal obstruction, hydrosalphinx and destruction of the fimbria, all of which can lower fertility. In the case of an IPAA, patients who underwent a laparoscopic intervention have a lower infertility rate than patients who underwent open surgery (6).

Preconception

Timely pre-conception counseling in patients with an active pregnancy wish has been shown to result in less relapses during pregnancy. This is related to medication adherence during pregnancy (7).

In addition to emphasizing the importance of medication adherence, it is advised to discuss the following factors during preconception counseling:

- The importance of a sustained remission of, at least, 6 months prior to conception
- Risk-benefit of current meds and possible adjustments
- Life style advise such as cessation of smoking, alcohol use and supplementation of folic acid
- Information about the heredity of IBD

- The effect of medication on breastmilk
- The mode of delivery as advised with regards to the disease location

Since disease activity increases the risk of relapse during pregnancy and negatively influences fertility, it is advised to strive for a (sustained) remission of approximately 6 months prior to conception (5,8). In the majority of patients, medication is needed to accomplish sustained remission and appropriate treatment of IBD should be maintained to reduce the risk of disease activity during pregnancy. To increase the adherence and correct usage of IBD medication during pregnancy, personalized consultation is of great importance.
General life style advice is also part of a preconceptional care and should include counseling about supplementation of folic acid, cessation of smoking and alcohol use.

An additional serious concern for IBD patients is the risk of their offspring developing the disease. Children of parents with IBD have an increased risk of developing IBD. When one parent is affected with IBD the overall risk for their children is 2-13 higher than the general population (9). When both parents are affected the risk becomes much higher, around 30% (10).
Further, mode of delivery and breastfeeding should be discussed during counseling. Active perianal disease is an indication for a cesarean section. Overall mode of delivery is subject to a multidisciplinary approach and primarily decided by an obstetrician on individual basis.

Pregnancy
During pregnancy, acute disease flares carry a high risk of adverse maternal and fetal outcome. Disease activity at time of conception and during pregnancy is associated with a higher rate of spontaneous abortion, preterm delivery, thromboembolic events, emergency caesarean section and low birth weight. According to current European guidelines, appropriate treatment of IBD should be maintained in those patients who wish to conceive, in order to reduce the risk of flares during pregnancy (5).

Medication during pregnancy
Aminosalicylates are considered low-risk medication during pregnancy (11). However, sulfalazine interferes with the resorption of folic acid which is important for women to take before and during the first 12 weeks of pregnancy. Therefore, it is advised to change to other IBD medication before pregnancy or increase the dose of folic acid to 2 mg/day (12). Medication containing dibutyl phthalate coating should be avoided during pregnancy (5, 13). Animal studies showed an increased risk of malformation in the male urogenital tract and a possible association with precocious puberty (13, 14).

Corticosteroids (CS) are used most often in the case of a disease relapse and can be used during pregnancy. CS cross the placenta, but the placenta is able to convert the medication into a less active metabolite by the enzyme 11-hydroxygenase. Studies show conflicting data regarding risk of malformations when mothers used corticosteroids during pregnancy. In one study the risk for orofacial malformations was increased in the children of mothers receiving corticosteroids in the first trimester (15, 16), but the risk was small and other studies did not report malformations (17-19). There also appears to be no risk of adverse pregnancy outcomes due to budesonide (20). Hydrocortisone, betamethasone and dexamethasone should be avoided, since they are longer-acting medications and less efficiently metabolized by the placenta.
Despite lack of evidence for malformation for CS use, there are reports of neonatal adrenal suppression when there is exposure to CS in utero (21). Therefore it is advised to consult a pediatrician who is able to examine the cortisol levels of the newborn.
The risk of maternal complications such as gestational diabetes and hypertension appears to be increases when using CS during pregnancy (22). Follow up by a gynecologist/obstetrician is therefore necessary.
Overall the use of corticosteroids during pregnancy is of low risk, but risks and benefits should be considered when prescribing this medication.

Thiopurines are considered of low risk and it is advised to continue these medications during pregnancy. Azathioprine and 6-mercaptopurine are converted into the active metabolite 6-TGN and 6-MMP. It was shown that 6-TGN crosses the placenta (23). Recent studies reported no adverse pregnancy outcomes for children exposed to thiopurines *in utero* (24-27). Limited data for 6-TG as a medicine is known, however it is transferred across the placenta.

Use of methotrexate (MTX) is prohibited. MTX is teratogenic and therefore contraindicated, at least 6 months prior to conception and during pregnancy (28). In the unfortunate case that MTX is not stopped before pregnancy the drug needs to be stopped immediately and high dose folate should be started. Further counseling with an obstetrician to discuss therapeutic abortion should be considered.

Biologicals (i.e. medication consisting of [humanized] proteins, for instance monoclonal antibodies) form the latest line of treatment for IBD. The most commonly prescribed biologicals are infliximab (IFX) and adalimumab (ADA), which target the pro-inflammatory cytokine tumor necrosis factor-alpha (TNFα). These two IgG1 antibodies cross the placenta in the second and third trimester of pregnancy (30). Although exposure to IFX or ADA does not seems to increase the risk of adverse pregnancy outcomes, the long-term effect on children who were exposed *in utero* remains unknown. Therefore, discontinuation of the treatment during pregnancy might be considered to limit this intra-uterine exposure. If the

disease is in sustained remission it is possible to stop these agents around week 20 of pregnancy. Stopping anti-TNFα therapy during second and third trimester does not increase the flare rate (31).
It is advised to measure cord blood levels of biologicals to determine the levels of these medications in the neonate. Anti-TNFα cord blood levels are dependent on the stop week of the anti-TNFα. However, when anti-TNFα is continued throughout pregnancy, the drugs levels of the infant will exceed the levels measured in the mothers (32). These high levels of anti-TNFα in the fetal blood do not seem to have an influence on the pregnancy outcome (33). In the first year of life there is also a normal growth and development of the children (34). The achievement of milestones in children exposed to anti-TNFα agents, thiopurines or combination therapy were similar or better than the cohort who was not exposed to medication (35). For monotherapy with anti-TNFα no increased infection rate in the infants has been reported. In the case of combination therapy with anti-TNFα and thiopurine there was a higher rate of infection in the infants (34). It is recommended to follow up drug levels every 3 months, until drug levels are undetectable. When levels are still detectable infants should be considered immune suppressed. Therefore administration of live vaccines should be avoided in the first 9 months of life and it is advised to not bring the children to daycare until drug levels are acceptably low. In addition to IFX or ADA, other biologicals targeting TNFα have recently become available. Of these, data on pregnancy outcomes of Golimimab are limited but because of the similarity of the safety profile to other anti-TNFα treatments it is considered of low risk. Certolizumab pegol (CZP) is transferred across the placenta by passive diffusion. Due to this mode of action, the levels of CZP reaching the fetus are probably much lower when compared to ADA and IFZ. No increase in adverse pregnancy outcomes was found when using CZP (36).

Other biologicals include Vedolizumab, which targets the T-cell adhesion receptor Integrin α4β7. Vedolizumab is an IgG1 antibody and will be actively transferred across the placenta during second and third trimester. The data on outcome of children exposed to vedolizumab is very limited. One report, limited by sample size and follow-up, showed the outcomes of 24 women exposed to vedolizumab during pregnancy, which identified no new safety concerns (37). Vedolizumab is gut specific which leads to the hypothetical concern that there might be an increased risk of gastrointestinal infections, such as rotavirus in the infants.
Ustekinumab has just recently been added as biological treatment medication for IBD, and targets the p40 subunit of the pro-inflammatory cytokines IL12 and Il23. It has been available for the treatment of psoriatic arthritis and psoriasis for several years and the experience of this medication in pregnancy is from case studies in psoriasis and psoriatic arthritis patients(38-41). No adverse pregnancy outcomes were noted in these cases. Since there is limited evidence, rheumatologists generally advice to consider alternative medications during pregnancy (42).

Thalidomide use has been associated with fetal malformations and a neonatal mortality rate of 40% (29). Therefore thalidomide is contraindicated during pregnancy.

Lactation

In general, breastfeeding is supported because there are many advantages for mother and child and it is not associated with a higher chance of relapse in women with IBD (43). It is known that IBD women breastfeed their babies for a shorter period compared to the general population (44). Reasons for this are concerns about the transfer of medication into the breastmilk and fatigue of the mother. In Table 1 the different IBD medications and their risks during lactation are shown. It is important to note that for some drugs there is limited data.

Aminosalicylates and corticosteroids are excreted into breastmilk, but are considered of low risk (45-47). Small concentrations of thiopurine have been detected in breastfed babies from mothers who were using thiopurines (AZA/ 6-MP), but there were no adverse outcomes detected in these children (48, 49). For 6-thioguanine (6-TG) this data is not available, but by mode of action it is considered of low risk.

Studies on IFX and ADA showed no impact on the infection rate in the infants, although the medicines are excreted into breastmilk in small quantities (50, 51).

So far only animal studies have reported on vedolizumab and ustekinumab. These medicines are considered of low risk by their mode of action (52, 53), but in the absence of sufficient data it is advised to not breastfeed infants when mothers take vedolizumab or ustekinumab.

Thus, the key elements in the consultation of IBD patients about pregnancy are:

- Women with quiescent IBD are as fertile as the general population
- All women of reproductive age should receive preconception counseling
- Conception occurring at a time of active disease increases the risk of persistent activity during pregnancy
- There is a higher risk of adverse maternal and fetal outcome in case of disease activity during pregnancy
- Appropriate treatment of IBD should be maintained in those patients who wish to conceive, in order to reduce the risk of flares during pregnancy
- Breastfeeding is possible for women with IBD (also when taking IBD medication), but data on transfer of medication into breastmilk is sparse and long-term studies need to be done.

Table 1. IBD drugs and their risk during lactation

Drugs		***Risk during lactation***
Aminosalicylates		
• Mesalazine	Very little excretion into milk	Low risk
• Sulfasalazine		Low risk
Corticosteroids		Low risk
• Prednisone		
• Prednisolone	Delay breastmilk for 4 hours	
• Budesonide	Very little excretion into milk	
Thiopurines		
• Azathioprine (AZA)	Undetectable or low levels	Low risk
• 6-mercapotpurine (6-MP e.g. Mercaptopurine)	Undetectable or low levels	Low risk Limited data available,
• 6-thioguanine (6-TG)		probably low risk
Methotrexate		High risk, do not use
Anti-TNF		
• Adalimumab	1/100 of the maternal drug levels detected	Low risk
• Infliximab	1/200 of the maternal drug levels detected	Low risk
• Thalidomide		Limited data available,
• Golimumab	Not detectable	probably low risk
• Certolizumab pegol		High risk, do not use
		Low risk
Vedolizumab		Limited data available, probably low risk
Ustekinumab		Limited data available, probably low risk

OUTLINE OF THE THESIS

As discussed in the introduction above, IBD and reproduction has been extensively studied in the past years. Since many women with IBD will at some time become pregnant, it is of great importance to broaden our knowledge regarding IBD, IBD treatment and the effect of pregnancy on IBD and *vice versa.*
The aim of this thesis is to extend this knowledge and approach the topic from both bench and bedside.

PART I BENCH
In the first part of this thesis, we go back to the basics and discuss the modulatory effect of changes during pregnancy on the course of IBD. Above it was emphasized that a stable disease is important for a healthy pregnancy. In order to obtain and retain disease remission it is important to understand the potential effect of pregnancy-induced changes on disease course. In **CHAPTER 2** we address the pregnancy-induced physiological changes and their potential effect on the disease course of ulcerative colitis and Crohn's disease, with emphasis on the modulation of epithelial barrier function and immune profiles by pregnancy hormones, microbial changes, and microchimerism. There is evidence suggesting a modulatory role of sex hormones on the intestinal barrier functions. But it remains unclear whether these hormones directly affect epithelial barrier cells. In **CHAPTER 3** we studied modulation of the epithelial barrier function, production of cytokines, and endoplasmatic reticulum (ER) stress, a hallmark of IBD, in response to sexhormones in organoids and colonic adenocarcinoma cell lines.
In **CHAPTER 4**, we extended some of these findings to the human situation. In addition to the hormonal changes and their effect we also studied immunological and microbial changes that take place in the female body during pregnancy. We showed that pro-inflammatory cytokines decrease upon conception in IBD patients and that IBD-associated dysbiosis in the microbiome seems to normalize to diversity levels seen in healthy pregnancy at trimester 2 and 3. Overall, it seems that immunological parameters improve in IBD patients on pregnancy, hormonal changes have a positive effect on epithelial barrier function and the microbial diversity normalizes to the levels seen during healthy pregnancy.

PART II BEDSIDE
Whether these positive changes last and translate to an altered disease course is unclear and there is a controversy regarding the effect of pregnancy on the clinical outcome. In **CHAPTER 5** we describe the effect of pregnancy on the disease course before and after a first pregnancy in patients with IBD.
CHAPTERS 2 to **5** focused primarily on the mother/IBD patient, but in order to give good counseling, the pregnancy outcome and health of the children that are born to these mothers is equally important. In the past years, most studies focused on the clinical health out-

come of children born to IBD mothers and these studies were reassuring. However, little is known about the effect of having IBD during pregnancy on the quality of life of these children. In **CHAPTER 6** we found, also reassuringly, that having IBD during pregnancy has no effect on the health-related quality of life of offspring in the first 5 years of life.

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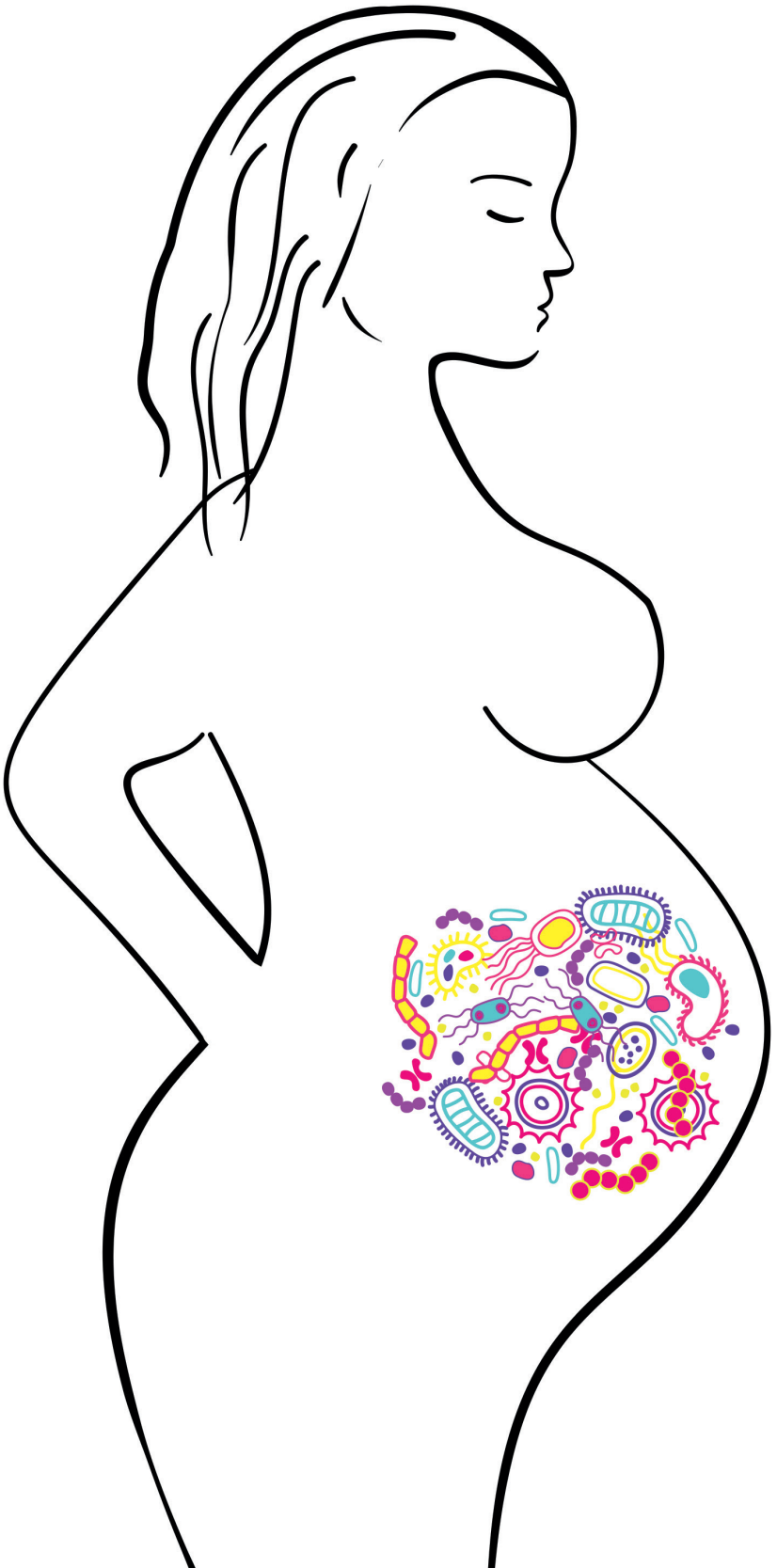
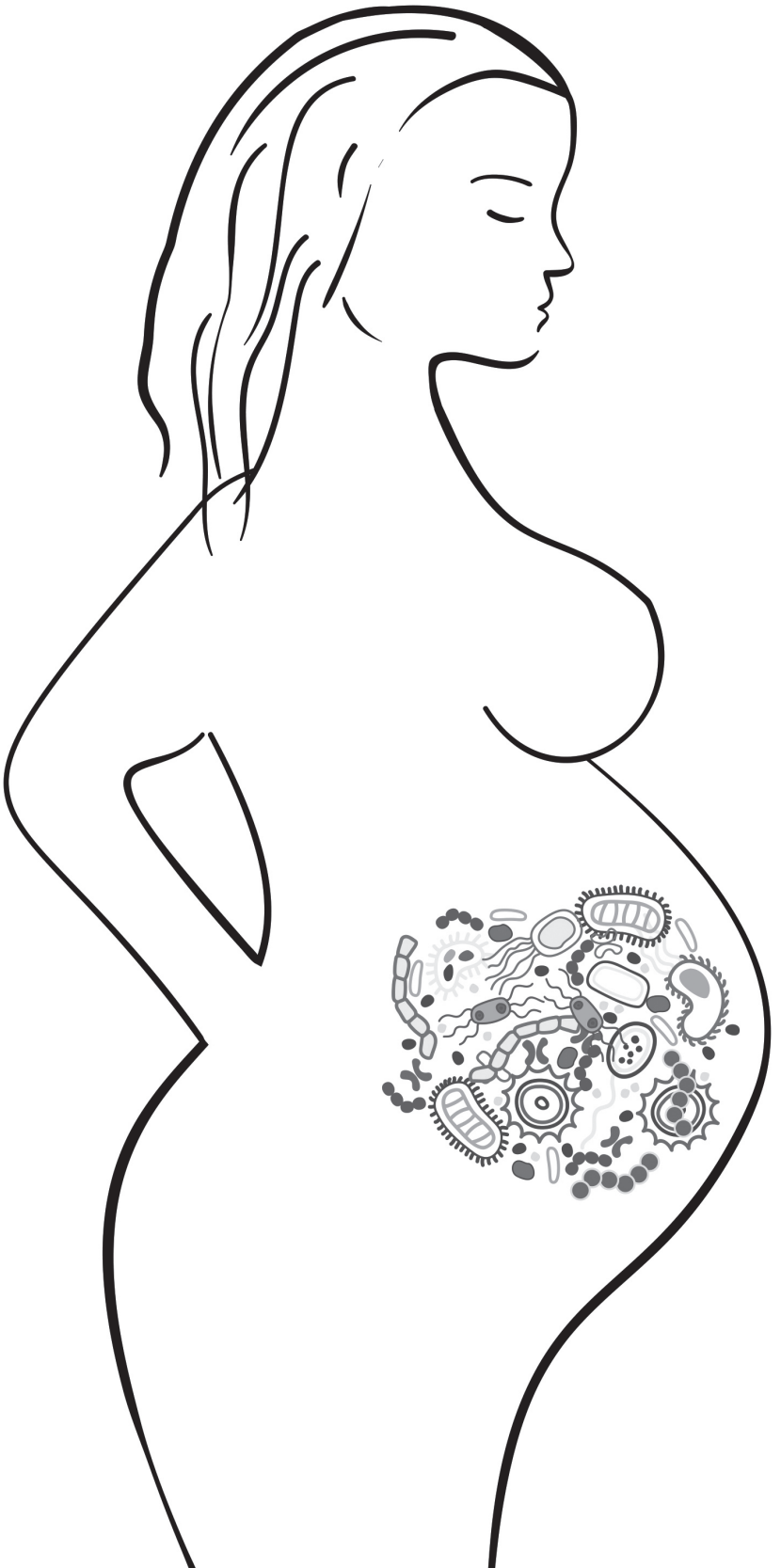


PART 1

BENCH



CHAPTER 2

Modulatory effect of pregnancy on inflammatory bowel disease

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ABSTRACT

The disease course of auto-immune diseases such as rheumatoid arthritis is altered during pregnancy, and a similar modulatory role of pregnancy on inflammatory bowel disease (IBD) has been proposed. Hormonal, immunological and microbial changes occurring during normal pregnancy may interact with the pathophysiology of IBD. IBD consists of Crohn's disease (CD) and ulcerative colitis (UC) and because of genetic, immunological and microbial differences between these disease entities, they may react differently during pregnancy and should be described separately. This review will address the pregnancy-induced physiological changes and their potential effect on the disease course of UC and CD, with emphasis on the modulation of epithelial barrier function and immune profiles by pregnancy hormones, microbial changes and microchimerism.

INTRODUCTION

Inflammatory bowel disease (IBD), comprised of Crohn's disease (CD) and ulcerative colitis (UC), is a group of chronic diseases of the gastrointestinal tract that affects men and women in their reproductive years of life. IBD and IBD therapies can have an impact on fertility, pregnancy outcomes, and fetal/neonatal health. *Vice versa*, the changes in hormones and in the immune system that occur during pregnancy may also influence IBD disease activity. There is a clear link between the female reproductive cycle and the gastrointestinal tract, as demonstrated by several studies reporting an increase in gastrointestinal symptoms among women with IBD and irritable bowel syndrome (IBS) before and during the menstrual period (1,2) and in changes to the menstrual function among women with IBD (3). Physiological changes that occur during the menstrual period include changes in hormones, cytokines and immune profiles, which may affect gastrointestinal motility, inflammation, and sensitivity (4). Similar changes also occur during pregnancy, and a modulatory role of pregnancy on inflammatory disease behavior has therefore been the topic of research for many years. The most convincing amelioration of (auto)inflammatory disease during pregnancy is observed in rheumatoid arthritis (RA), where symptoms abate during pregnancy, and flares are commonly observed post-partum (5,6). With many of the underlying pathogenic mechanisms (genetics, intestinal microbiome alterations, immune shifts) overlapping with IBD, resulting in several shared treatment options (7–9), it is not surprising that a disease modulatory role for pregnancy in IBD has also been speculated upon. Nevertheless, conflicting results of the effect of pregnancy in IBD have been observed. One study showed that both CD and UC patients experienced fewer flares in the 3 years post-partum as compared to their flare-rate before pregnancy (10). A 10 year follow-up study confirmed that relapse rates decreased in UC (from 0.34 to 0.18 flares/year) and CD (from 0.76 to 0.12 flares/year) following pregnancy (11). In addition, it appears safe to stop anti-TNFα treatment in pregnant IBD patients, without increasing the risk of flares (12,13). However, these data are disputed by a study of Pedersen *et al*, who showed that pregnant women with CD have a similar disease course during and after pregnancy as compared to non-pregnant women with CD. In contrast, women with UC have a higher risk of relapse during pregnancy (relative risk [RR] 2.19) as well as postpartum (RR 6.22), compared to non-pregnant women with UC (14). The course of IBD disease activity during pregnancy is closely related to disease activity preconception (15), with women who conceive during a time of active disease having twice the risk (RR 2.0) of disease flare during pregnancy compared to those who conceive during a time of remission. While the often reported medication non-adherence during pregnancy may be a confounding factor (16), disease course during pregnancy appears to be related to the type of IBD, suggesting a true relationship between pregnancy and disease activity.

In this review, we summarize the current knowledge regarding the interaction between reproductive physiology and IBD pathophysiology, and propose explanations for the clinical observations of IBD disease behavior during the reproductive period. We describe the pathological alterations in barrier function, immunology and microbiome in IBD and discuss how these factors are modulated during pregnancy. A better understanding of these complex interactions and clinical observations will aid clinicians and researchers in improving the management of IBD during pregnancy, and optimize maternal and neonatal outcomes.

PATHOGENESIS OF IBD

IBD is a multifactorial disease, in which an altered immune response towards the intestinal microflora results in chronic inflammation of the intestinal tract. In addition to environmental factors (hygiene, smoking, diet etc), genetic susceptibility plays an important role in IBD, and large genome wide association studies (GWAS) have identified over 200 genetic loci associated with an increased risk of developing IBD (17,18). Interestingly, attempts at identifying common underlying mechanisms based on these loci have uncovered an important role for (innate) immunity and bacterial handling in IBD susceptibility: many of the identified risk genes can be classified in pathways affecting epithelial barrier function, innate immune cell function or adaptive immunity. All of these processes are critical at the contact interface between host and bacteria, underscoring the importance of these interactions in IBD development (18).

Epithelial barrier function in IBD

The first obstacle for bacterial invasion is represented by the intestinal epithelial barrier, which, although not traditionally regarded as part of the immune system, is now gaining recognition as part of the first line innate immune defense. Bacteria are physically separated from the actual barrier cells through the production of a mucous layer and the release of anti-microbial peptides therein by goblet cells and Paneth cells, respectively. Disease predisposing genetic variants in mucin genes may contribute to alterations in the mucus layer in IBD patients (19). With the mucosal layer breached, bacterial components have an increased chance to reach the epithelial cell layer. In response, different immune cells at the mucosa/luminal interface produce inflammatory cytokines, such as interferon-gamma (IFN-ɣ) and tumor necrosis factor-alpha (TNF-α), which can inhibit anti-apoptotic proteins and promote apoptotic processes, resulting in a weakening of the epithelial lining and an increased translocation of pathogens (20). This process, referred to as ‘leaky gut’, is already seen in healthy first degree relatives of patients and therefor appears to one of the disease initiating events in IBD patients. Barrier dysfunction is worsened during active disease, when there is an additional reduction in tight junctions, which regulate the epithelial permeability (21). Thus, the overall weakening of the barrier function in IBD results in an

enhanced exposure of the mucosa to bacterial components, which stimulates the attraction of immune cells and perpetuates inflammation.

Epithelial barrier function during pregnancy

Female reproductive hormones fluctuate during the normal menstrual cycle, with estrogen reaching peak levels prior to ovulation and progesterone reaching peak levels during the luteal phase of the cycle. Fluctuations of these hormones even on estrus scale already appear to affect bowel health. The gut epithelium expresses receptors for both estrogen (estrogen receptor [ER]α and β) and progesterone (22), and data in animal models show that paracellular permeability is decreased during the estrogen dominant phase of the cycle as compared to the progesterone dominant phase, consistent with an improved barrier integrity in response to estrogen (23). Gut epithelial cells in female rats are also more resistant to injury and inflammation than in male rats, and application of estrogen to male gut cells abrogates the enhanced inflammatory susceptibility in these male cells (24). Furthermore, progesterone receptor expression is increased in constipated persons, suggesting that even though progesterone does not have a direct effect on barrier integrity (23), it may affect ion and water transport in the gut (25).

With these relatively small systemic fluctuations in hormone levels already impacting epithelial barrier function in an anti-inflammatory and diarrhea-reducing manner, it is tempting to speculate that similar actions on a larger scale take place during pregnancy. Estrogen and progesterone levels increase rapidly during the first trimester, causing some of the nausea women experience. Estrogen peak levels are reached during the third trimester, accounting for the vascularization of the placenta and uterus, supporting the development of the fetus and the development of the milk duct. Interestingly, while low levels of 17-beta-estradiol decreased paracellular permeability of vascular endothelial cells, high levels of estradiol increased the permeability, as a result of biphasic modulation of the tight junction molecule occludin (26). Thus, while epithelial barrier function has not been investigated during pregnancy it is possible that studies performed during estrus are not reflective of a protective role of estrogen during pregnancy. Nevertheless, in IBS a link between increased gastrointestinal symptoms at the lowest estrogen levels of the menstrual cycle and reduced complaints during pregnancy is suggestive of a positive effect of pregnancy hormones on intestinal health (27).

Immunity in IBD

Innate immunity

The immune system represents a complex interplay of different cell types aimed at defending the human body from pathogenic microorganisms. Innate immunity is the first line defense of the body against infections and includes **monocyte/macrophages, granulocytes, and dendritic cells (DCs)** (Table 1). These cells, constitutively present in body tissues, act as sentinels of the body by indiscriminate uptake (phagocytosis) and digestion

Table 1. Changes of the immune system during pregnancy and IBD

Table 1	Cell type	Sub-type	Function	Changes in IBD	Changes during pregnancy	Conceivable effect of changes during pregnancy on IBD
Epithelial barrier	Goblet cells		Production of a mucous layer	Decrease of mucus layer in IBD	Improved barrier integrity in response to estrogen	Positive effect, mainly due to increase estrogen
	Paneth cells		The release of anti-microbial peptides	Decrease of anti-microbial peptides in IBD		
Innate immune system	Monocytes	Macrophages Dendritic cells (DCs)	• Phagocytosis and digestion of pathogens • Antigen presentation and activation of the adaptive immune system • DCs express IDO1, which induces apoptosis of CD8+ T-cells and promotes differentiation of CD4+ T-cells to Tregs	• Increased in IBD • Skewing of macrophages from M2 (important for wound healing) to M1 (inflammatory) phenotype	• Decrease from early pregnancy to mid-gestation • Skewing towards M2 wound healing macrophages at placental interface • Fetal tolerance via Tregs	Positive effects through circulating M2 macrophages and Tregs and their cytokines
Adaptive immune system	Granulocytes		• Phagocytosis and digestion of pathogens • Release a number of different effector molecules at site of infection	Increased in IBD	Decrease from early pregnancy to mid-gestation	Decrease during pregnancy may have a positive effect on IBD course
	Natural Killer (NK) cells		• secrete cytokines such as IFNγ and TNFα, which act on macrophages and DCs • ability to kill tumor cells without any priming or prior activation	Increased in CD	Increase of placental and decidual NK cells	Local effect during pregnancy, so probably no influence on the course of IBD
	Innate Lymphoid cells (ILCs)	ILC1 (INF-γ, TNF-α) ILC2 (IL-4, IL-5, IL-9, IL-13) ILC3 (IL-17, IL-22, TNF-α)	Secrete immunoregulatory cytokines	• ILC1 increased in CD • ILC2 increased in IBD • ILC3 increased in IBD	First trimester: increase of ILC1 and ILC3	Negative effect on CD, potentially beneficial effect on UC
	T-cells	T-helper 1 (Th1) (IL-2, IL-12, INF-γ, TNF-α)	Cytotoxicity, antitumor and antiviral responses	Increased in CD	First trimester: increased in local tissue	UC more likely to flare during pregnancy

		T-helper 2 (Th2) (IL-4, IL-5, IL-6, IL-9, IL-10, IL-13)	Antibody mediated immunity by stimulating B cells	Increased in UC	Second/third trimester: increase in local tissue	CD, not UC, may benefit from the shift to Th2 phenotype
		T-helper 17 (Th17) (IL-17, IL-21, IL-22)	Protect cell surfaces by removing extracellular bacteria	Increased in UC		
		Regulatory T-cells (Tregs) (TGF-β, IL-35, IL-10)	Regulate the function of other T-cell subsets and thereby repress inflammatory processes	Increased in IBD	Second trimester: increased	Unclear
Hormones	HCG		• Mediates early expansion of Tregs • Modulates DC responses by inducing IDO-1 expression • Reduces inflammatory cytokines such as IL-17 while increasing IL-10 levels • In vitro, HCG is able to stimulate peripheral blood DC subsets to maintain a tolerant phenotype		Increase in first trimester	HCG or its peptides may contribute to the amelioration of inflammatory processes
	Progesterone		• Decrease of pro-inflammatory mediators (ie TNF-α, IL-6, IL1-β, NO) • Increases IL-10 production by macrophages and monocytes		Increase during pregnancy, with a decrease before labor	Conflicting data
	Estrogen		• Decreases inflammatory cytokine production (i.e. TNF-α, IFN-ɣ) • Inhibits NO synthase activity • Decreases the recruitment of inflammatory cells		Increase during pregnancy	Conflicting data in animal studies and human studies regarding contraceptive use
Microbiome	Bacteria and their metabolites			• Reduced diversity of the bacteria present • Decrease of anti-inflammatory Firmucutes (ie F. Prausnitzii) • Increase of proteobacteria and Bacteroidetes phylym members • CD show a less stable microbiome and reduced diversity compared to UC patients	Third trimester: dysbiosis, resembling a state of low-grade inflammation of the gastrointestinal tract	Worsening of IBD because of increase of dysbiotic changes seen in IBD during pregnancy
Micro-chimerism	Immune cells, stem cells		Co-existence of two genetically different populations of cells in one individual	• Maternal microchimerism is not increased in IBD patients • Fetal microhimerismin IBD pregnancy remains to be investigated	Whole cells cross the placenta from mother to child and vice versa	Fetal microchimerism in IBD pregnancy remains to be investigated, but adverse effects speculated in (auto)immune diseases

of pathogens. Increased numbers of granulocytes, macrophages and (DCs) have been observed in intestinal lesions in IBD. These cells may contribute to exacerbation of disease by releasing damaging reactive oxygen species and increasing local pro-inflammatory cytokine levels. An inherent alteration in bacterial responses of these cells appears present in IBD, which may contribute to the pathogenesis (28–30). For instance, macrophages from IBD patients show increased pro-inflammatory and decreased anti-inflammatory cytokines when stimulated with bacteria (31). Epithelial wound healing in colitis models requires the presence of specialized M2 macrophages (32,33), which, in contrast to pro-inflammatory M1 macrophages, release anti-inflammatory IL10, and contribute to tissue remodeling. In mucosa from patients with IBD, a shift towards M1 macrophages at the expense of M2 macrophages is observed (34), which may contribute to an impaired mucosal healing and prolonged inflammation.

Adaptive immunity

Presentation of pathogenic antigens on the cell surface of DCs and macrophages cells in the context of major histocompatibility complex (MHC) II molecules can subsequently activate cells of the adaptive immune system, in particular CD4+ T helper cells (Th cells). Upon antigen stimulation T-cells differentiate into different subsets, depending on the local cytokine milieu. These include **Th1, Th2, Th17 and regulatory T-cells (Tregs)**, which each fulfill different functions and produce different cytokines. Distinct cytokine expression differences as well as T-cell subset activities have been observed between CD and UC patients (35), and an over-activation of the adaptive immune response with mucosal infiltrating T-cells is evident, with the effectivity of targeted therapies against T-cells underscoring the importance of this cell compartment in disease activity. While IFN-ɣ and Interleukin (IL)-17A cytokine expression, representative of Th1 and Th17 cells respectively, are increased the lamina propria in CD, the Th2 cytokines IL-4, IL-5 and IL-13 are increased in UC (36). Under normal circumstances Th1 and Th2 cells are in a dynamic equilibrium, with an imbalance resulting in either Th1 or Th2 dominant diseases. While a gross simplification, CD is now generally regarded as a Th1/Th17 disease, whereas UC is considered a Th2/Th17 disease.

Tolerance

Of course, with the number of bacteria being equal to the number of human cells in the body (37), it is imperative that the immune system does not respond to all bacteria present. Immune tolerance development is therefore key to a successful symbiosis with our commensal microflora, and is largely mediated by Tregs which suppress T-cell activation via production of cytokines such as IL-10 and transforming growth factor (TGF)-β (38). In addition, upon IFNγ stimulation, DCs express the enzyme indoleamine 2,3,-dioxygenase (IDO1) which converts the essential amino acid tryptophan into kynurenine. This has the dual effect of inducing apoptosis of CD8+ T-cells by tryptophan depletion, and skewing CD4+ T-cells to Treg differentiation (39,40). While theoretically it might be expected that regulatory T cell

functions would be decreased in intestinal inflammation, the reverse has been observed: in IBD, both the number of Tregs as well as their differentiation-inducing agent IDO1 are increased in mucosal IBD biopsies compared to non-IBD patients (41,42). In part, this seems a (failed) compensatory mechanism, with Tregs from the peripheral blood being recruited to inflamed mucosal area (42). Nevertheless, many experimental models show benefit of redirecting the Th/Treg balance, and suggest that Tregs may be a suitable target for treatment (43). Phenotypic alterations associated with reduced tolerance induction have also been observed for DCs and macrophages in IBD (44,45).

Innate lymphoid cells

In addition to innate myeloid cells, a family of lymphoid-derived cells with innate properties exists, which includes **Natural Killer (NK) cells** and a relatively recently identified subset of cells called **innate lymphoid cells (ILCs)** (46) (Table 1). These cells are enriched at the intestinal mucosa, but unlike 'real' lymphocytes, do not require antigen recognition in MHC-II context, but rather rely on myeloid-derived cytokines and natural cytotoxicity receptors for their activation(47). Increased numbers of IL17 and INF-γ producing NK cells and ILCs have been observed in mucosal biopsies from CD (48,49), but not UC patients (50). While several experimental models have now highlighted the importance of ILCs for IBD pathology (reviewed in (51)), and NK cell populations are a target for treatments such as 6-MP and azathioprine (48,52), cytokine disturbances in IBD have traditionally been linked to a skewing in adaptive immune responses, in particular those represented by Th-cell subsets.

Immunity during pregnancy

Many excellent reviews have already been written on the immunological changes taking place during pregnancy (53–56), the main findings of which are summarized here.

During pregnancy, an MHC mismatched fetus is present in the mother, which, despite the presence of a placental barrier still affects the maternal immune system. Thus, induction of tolerance against paternal antigens appears to lay at the heart of immunological changes in successful pregnancies. Immune cells infiltrate the placenta during pregnancy, around 70% of which consists of **NK cells**. Unlike peripheral NK cells, placental NK cells are not cytotoxic, but help decidualisation, angiogenesis, immune tolerance and fetal development by producing growth factors (57,58). Decidual NK cells may possess both immune activating and regulatory properties (59), and while their presence is beneficial during early pregnancy, their persistence or failure to switch to a different phenotype in later pregnancy is associated with adverse pregnancy outcomes (60,61).

The remainder of placental immune cells consists mostly of **macrophages and T-cells, including Tregs**. Macrophages in the decidua show a distinct M2 phenotype, and are a major source of placental anti-inflammatory IL-10 and show reduced T-cell activating properties compared to their peripheral blood counterparts (62). They (as well as DCs and trophoblasts) are an important source of the soluble IDO1 enzyme, which contributes to

the generation of Tregs and establishment of fetal tolerance (63). It has been postulated that a shift from inflammatory Th1 to more permissive Th2 cytokine profiles is required for a successful pregnancy (64,65). IL-25, an IL-17 family member expressed by decidual T-cells, NK cells, Tregs and macrophages, stimulates the production of IL-4 and IL-10 in decidual T-cells, thereby contributing to a Th2 environment in first term placentas (66). Furthermore, human term placentas show increased levels of Th2 cytokines compared to preterm placentas (67,68). However, it is increasingly accepted that a healthy pregnancy depends on the maternal immune system to adapt to the different stages of pregnancy, and that pro-inflammatory processes are also required for the tissue remodeling which is essential for decidua formation as well as labor induction (53). For instance, despite the presence of IL-10, the first trimester of pregnancy is also characterized by the **presence of a pro-inflammatory Th1 immune profile** for the successful implantation of the blastocyst and IL-6, IL-8 and TNF-α are present at the implantation site (55). The source of these cytokines may be Th1 cells (69), although ILC1 and specialized ILC3 cells have also been observed in first term placentae (70). Cell subsets shift during pregnancy, with the presence of macrophages declining from early to mid-gestation, while T-cell frequencies increase during this time interval (71). Term labour and delivery appears to require low level, well controlled inflammatory processes (72). Correspondingly, placental IL10 levels decrease towards labor (73), and rat models indicate an increase IL-6, TNFα and IL-1β in term placentas (74).
In toto, the current general consensus suggests that implantation requires a Th1 response, followed by a shift towards a Th2 phenotype for the main duration of pregnancy, and again a Th1 milieu towards partition (75). It should be noted however, that much of the data comes from animal studies, which may not necessarily reflect the human situation as in contrast to human placentas, T-cells represent a rare population in mouse placentas (76).

Systemic immunological effects of pregnancy on IBD?

Differences in disease behavior between Crohn's disease and ulcerative colitis during pregnancy and peripartum may potentially be explained by intrinsic differences in the immune pathways that lead to each disease. As seen above, pregnancy is associated with immunological changes at the fetal/maternal interface, with a predominantly Th2/tolerogenic phenotype. Thus is it tempting to speculate that a Th2 shift during pregnancy ameliorates disease in those patients in whom Th1 responses dominate (such as CD), while aggravating disease in Th2 dominant patients (mainly UC). Nevertheless, the maternal peripheral immune system is still capable of mounting a robust immune response to pathogenic antigens (77), and the question therefor remains to what extent placental immunological changes can affect immunological processes at distant body sites.
Levels of Th1 and Th2 patterns *in utero* generally appear to be mirrored by ratios in peripheral blood (53), although most data are derived from studies comparing pregnancy outcomes, and hence blood is usually obtained at only one timepoint, often post-partum. There are conflicting data on modulation of serum cytokine levels in healthy pregnant women, with

some studies reporting a significant decrease of pro-inflammatory Th1 cytokines (e.g. IL-8, IL-12, IFN-γ, TNF-α) from first to third trimester in healthy pregnant women (78), and others showing no difference or even an increase (79,80). For Th2 cytokines even less is known, with one study reporting a stable level of IL-4 and IL-5 during pregnancy (81). Thus, it is unclear to what extent pregnancy induces peripheral cytokine changes which may influence inflammatory diseases. However, ample evidence suggests that peripheral blood cell subsets at least are altered in normal pregnancy. For instance, stimulated peripheral blood mononuclear cells from pregnant women produce less Th1 and Th2 cytokines compared to healthy controls, in particular during second trimester, while levels increased post-partum, suggesting that systemic alterations in cell sensitivity exist during pregnancy which may contribute to decreased (auto-)immunity during pregnancy and increased flaring thereof, afterwards (82). The peripheral blood percentage of Tregs also peaks during the second trimester of pregnancy, and *in vitro*, these Tregs are capable of reducing T-cell activation in response to DCs (83). As development of Tregs during pregnancy appears related to the presence of fetal alloantigens rather than pregnancy hormones(84) and the Treg recognition receptor repertoire differs per organ (85), it is uncertain to what extent pregnancy-induced circulating Tregs would be useful at the inflamed mucosa. Nevertheless, much is unclear regarding mucosal Treg antigen recognition (86) and the fact that peripheral blood Treg levels drop during inflammation suggest that general recruitment of Tregs to inflammatory sites occurs(42). Their presence there may potentially contribute to modulation of inflammatory processes through production of inhibitory cytokines or suppression of DC maturation.

Similar to Tregs, NK cells present in pre-implantation endometrium show a different receptor repertoire compared to peripheral blood NK cells in the same women (87). However, it has also been reported that in the first trimester of pregnancy, progesterone-dependent expression of the receptor TIM-3 on peripheral blood NK cell confers immunosuppressive properties (88), and it is conceivable that these cells also reach the intestinal mucosa where they may modulate disease activity.

Systemic effects of pregnancy hormones

The most important early immune modulator in pregnancy is now acknowledged to be human chorionic gonadotropin (hCG), which mediates early expansion of regulatory T-cells (Tregs), modulates DC responses by inducing IDO-1 expression, and reduces inflammatory cytokines such as IL-17 while increasing IL-10 levels (89). Importantly, many of these effects occur in peripheral blood from non-pregnant patients receiving hCG for their IVF treatment. *In vitro*, hCG is able to stimulate peripheral blood DC subsets to maintain a tolerant phenotype (90). Several cleaved or 'nicked' forms of hCG exist *in vivo*, and studies have shown that such hCG peptides show anti-inflammatory properties in a host of mouse models, including LPS-induced septic shock, polymicrobial sepsis, haemorrhagic shock and diabetes (91,92)(93–96). Administration of hCG peptides also inhibited neutrophil recruit-

ment and inflammatory markers such as IL-6 and TNFα (96,97) (89). The same authors also showed that human graft versus host disease at the skin was successfully treated with hCG, which corresponded with increased IDO1 expression in peripheral mononuclear cells, as well as IL10 serum levels and Treg upregulation (98). With hCG administration being able to prevent auto-immune diabetes in mice by downregulating Th1 responses (99), the use of hCG to control the autoimmune processes in Rheumatoid arthritis and Sjogren's disease has been suggested (100), and it is tempting to speculate that hCG may also positively affect IBD.

The high amount of progesterone throughout pregnancy not only results in the laxity of the ligaments and joints, but is also thought to suppress the maternal immunologic response to fetal antigens and allow implantation in the endometrium. Progesterone reduces pro-inflammatory mediators (ie TNF-α, IL-6, IL1-β, nitric oxide [NO]) and increases IL-10 production by macrophages and monocytes (101). Application of progesterone in a temporomandibular joint inflammation model of ovariectomized rats reduced synovial inflammation and levels of TNF-α and IL1-β(102). In a rat colitis model, progesterone ameliorated disease activity through reduction of TNFα levels in colon and blood (103). Nevertheless, conflicting data have also been reported. In a chemically induced model of colitis (TNBS; 2,4,6-trinitrobenzene sulfonic acid), the progesterone dominant luteal phase of the menstrual cycle was associated with increased severity of colitis and treatment of ovariectomized animals with progesterone similarly increased disease severity, while estrogen decreased colitis(104). Indeed, anti-inflammatory properties have often been ascribed to estrogen, since it decreases inflammatory cytokine production (i.e. TNF-α, IFN-ɣ), inhibits NO synthase activity and decreases the recruitment of inflammatory cells (105,106). However, animal studies of IBD and the effect of estrogen also show inconsistent findings. Improvement of stool scores in HLA-B27 transgenic rats with chronic diarrhea was noted after treatment with 17α-ethynyl-17β-estradiol (EE) (107). Similarly, estrogen reduced TNFα, IL1β and IL-6 levels as well as inflammation in diverse rat models of colitis (108). Verdu *et al* found that a supraphysiological dose of 17β-estradiol has an anti-inflammatory effect in a dextran sodium sulfate (DSS) murine model for colitis but a pro-inflammatory effect in the dinitrobenzene sulfonic acid (DNB) colitis model (109). Clinical human studies of IBD and sex hormones focus mainly on postmenopausal women and/or oral contraception use. Kane *et al* described a protective effect of estrogen on the bowel in women with IBD (110), while Khalili *et al* showed that postmenopausal women who use oral contraceptives had a higher risk of developing UC, but not CD (111). A meta-analysis of Cornish *et al* demonstrated that with time of exposure to oral contraceptives the risk of developing CD was increased, and when contraceptives were stopped the risk decreased again to that of the normal population (112).
It is clear that there is conflicting data on IBD and levels of sex hormones and that it is difficult to translate these clinical studies to the situation in a pregnant IBD patient. Different

immune cells may react in an opposite manner to different concentrations of estrogen and progesterone, and expression patterns of receptors of these hormones may vary under inflammatory conditions, precluding robust predictions on the overall effect of progesterone and estrogen on auto-immune disease.

MICROBIOME IN IBD AND PREGNANCY

As mentioned before, IBD is thought to arise in consequence of an altered immune response towards intestinal bacteria. We now know that the microbiome of IBD patients is substantially altered as compared to healthy controls, and that inflamed regions show further microbial deregulation as compared to non-inflamed regions (113–115). This so-called dysbiosis includes a reduced diversity of the bacteria present, in particular in CD patients, with a noted decrease of anti-inflammatory *Firmucutes (i.e. F. Prausnitzii)* and an increase of proteobacteria and *Bacteroidetes phylym* members (*i.e. Bacteoides fragilis*)(116–119). The host-microbiome interaction is reciprocal, and it is as yet unclear whether dysbiosis in IBD presents the chicken or the egg in the etiology of disease. Nevertheless, the general consensus now favoures a causative role for the microbiome in disease initiation, as animal models indicate that bacterial presence is required for colitis development and that colitis may be conferred by transplantation of inflammation-associated feces (120).

Pregnancy is also accompanied by intestinal microbial changes. These changes induce a metabolic state that may be beneficial during pregnancy, as concluded by Koren *et al.* (121). They described that, in the first trimester of pregnancy, the gut microbiota is similar in many aspects to that of healthy non-pregnant controls. However, in the third trimester a dysbiosis was observed, resembling a state of low-grade inflammation of the gastrointestinal tract. This dysbiosis was accounted for by the presence of Proteobacteria and Actinobacteria and was not related to BMI (before pregnancy), antibiotic use, diet or the presence of gestational diabetes. The overall diversity of the bacteria was also reduced at T3. These data would suggest that microbial changes that occur during normal pregnancy fortify dysbiotic changes seen in IBD, and would aggravate disease activity. Interestingly, patients with CD show a less stable microbiome and reduced diversity compared to UC patients (122), and it is conceivable that further alterations during pregnancy therefore have less of an effect on CD disease activity as compared to UC. Of note, there are several studies which show that diet shapes the microbiome, and in particular western diets are associated with IBD (123,124). As it is commonly appreciated that women change their diet during pregnancy, it is important to take this into account in future studies.

It is clear that while microbial changes during pregnancy and the host defense mechanisms are both changed during pregnancy and IBD, the interactions are complex, reciprocal and double edged, hampering interpretations of the observed changes.

MICROCHIMERISM AND A-FETOPROTEIN

The placental exchange of maternal and fetal gasses, nutrients, metabolic waste products and antibodies is well described. However, in addition to these small molecules, it is also possible for whole cells to cross the placenta from mother to child and vice versa. Such co-existence of two genetically different populations of cells in one individual is termed (micro)chimerism. Cellular transport is bi-directional (125,126), with maternal cells detected in 24-42% of fetal-derived samples, and fetal cells were detected in 26-51% of mothers (127,128). Fetal cells, which can be detected as early as 7 weeks gestation, are known to persist for some time after delivery (129). In fact, microchimerism has been observed in mothers up to 38 years after pregnancy, and in offspring well into adult life (130,131). In addition to fetal mature T-cells, $CD34^+$ progenitor cells enter the maternal bloodstream during pregnancy, which retain their multi-lineage potential and can become adult hematopoietic cells of all linages as well as epithelial cells (131,132). With the potential for these cells to assert effector functions and affect the maternal immune system, the functional immunological consequences of these microchimers in health and disease is gaining interest (133,134). Male fetal cell-derived T-cell clones isolated from parous women show proliferation and IL-4 production in response to *ex vivo* stimulation with maternal T-cells and MHC antigens, and this effect was higher in patients with systemic sclerosis, suggesting that these offspring T-cells show a Th2 profile and could play a pathogenic role in immune disease (135). Interestingly, increased microchimerism was observed in peripheral blood mononuclear cells from patients with the autoimmune disease scleroderma, which has a peak incidence in women after childbearing years, again suggesting that such microchimerism may contribute in autoimmune disease (131). However, patients with either Grave's disease or Hashimoto's thyroiditis, two other autoimmune diseases associated with pregnancy, have reduced microchimerism as compared to healthy controls (136). Furthermore, microchimerism has been investigated in systemic lupus erythematosus (SLE), Sjogren's syndrome, Multiple sclerosis and RA, and may be either protective or harmful (133). In RA, where there is a clear beneficial effect of pregnancy on disease course, it had been suggested that accumulation of fetal T-cells, which appear around gestational week 13, may dampen the maternal immune response, and that this effect weakens over time, due to senescence of these cells (137). The presence of fetal cells in maternal tissues correlates to the presence of maternal Tregs, which may account for some of the dampening of inflammatory disease during pregnancy (134). While the exact effect of microchimerism on (auto) immune disease is as yet unclear, it is tempting to speculate that it may also play a role in IBD and affect disease course during pregnancy. Thus far, only maternal microchimerism has been studied in IBD, which did not appear to be increased in IBD patients (138,139). Fetal microchimerism in IBD pregnancy remains to be investigated.

Another potential fetal source contributing to maternal (auto) immune response is α-fetoprotein (AFP), a protein produced by the yolk sac and fetal liver, which can be detected

in the maternal serum from week 14 of pregnancy onwards. AFP was shown to bind to autoantibodies produced in patients with the auto-immune disorder myasthenia gravis, and it was thus speculated that circulating levels of AFP in the second and third trimester of pregnancy could induce clinical remission in MG patients during these times (140). The immunomodulatory effects of AFP extend beyond antibody binding (141). Studies indicate that AFP ameliorates a mouse model of MS, through, amongst others, inhibition of Th1 cytokine production (142). It has been speculated that AFP may be used for the treatment of MG, MS, autoimmune uveitis and psoriasis (143). Thus far, however, the potential role of AFP in IBD remains unexplored.

MODULATION OF IBD RESPONSE TO MEDICATION THROUGH PREGNANCY

Disease activity in women with IBD may also be modulated by pharmacokinetic changes induced by pregnancy and through interaction of IBD medication with the placenta which may modulate the clinical effectivity of these drugs. However, while drugs such as the thiopurine 6-thioguanine nucleotide (6TGN) and 5-aminosalicylic acid (5-ASA) are known to cross the placenta, this does not seem to influence therapeutic levels in the mothers (144). Less is known about the effects on patient and child outcomes of biologicals, the most recent IBD medications. The earliest of these are the anti-TNFα treatments (infliximab, adalimumab, golimumab, certolizumab pegol), with vedolizumab (α4β7-integrin blocker) and ustekinumab (IL12/IL23 blocker) following suit. From week 20 onwards, maternal immunoglobulins (Igs) are transported across the placental barrier, to provide immunoprotection to the fetus (145). Transport of Igs is mediated by the neonatal fragment crystallizable (Fc) receptor (FcRn) which binds to the Fc region present in all antibodies, including therapeutic monoclonal antibodies. Certolizumab pegol does not undergo this FcRn-mediated transfer across the placenta, because it lacks an IgG Fc region and therefore does not bind FcRn. Due to the passive diffusion across the placenta the levels of certolizumab pegol reaching the fetus are probably much lower when compared to infliximab and adalimumab (146). We and others have previously shown that infliximab and adalimumab levels in cord blood exceeds levels present in serum from mothers treated with these medications (147,148), suggesting that active transport of these antibodies over the placental barrier may decrease bioavailability of the antibodies in the mother. As serum drug levels of these therapeutic antibodies correspond to clinical outcomes for IBD patients, modulation of these levels through placental transport could potentially result in disease relapse (149,150). Thus far, however, maternal infliximab levels during pregnancy were shown to be increased, whereas Adalimumab levels remained stable. Nevertheless, pharmacokinetic changes of these therapeutic antibodies upon pregnancy have only been studied in a small cohort of patients, and larger studies are needed (151).

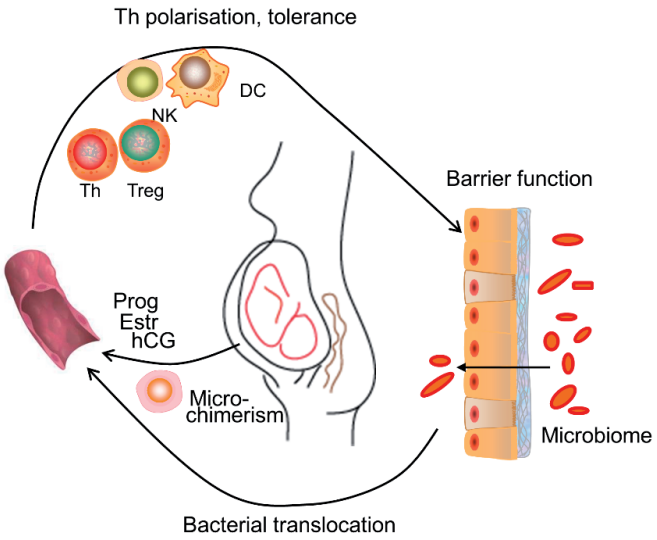

Figure 1. Interaction between physiological changes during pregnancy and the pathophysiology of inflammatory bowel disease.

SUMMARY

The observation that the disease course of several (auto)immune diseases are altered during pregnancy suggests that there is an interaction between physiological changes taking place during pregnancy and pathophysiology of these diseases (Figure 1). For IBD, this relationship appears more apparent for UC than CD, which may be due to the fact that CD and UC have differential underlying genetic susceptibilities, immune profiles and microbial changes. Genetic variants affecting cellular innate immunity are more associated more with CD, while UC-specific SNPs affect epithelial barrier function genes (152). Furthermore, CD patients show a more Th1 dominant cytokine profile and less stable microbiome compared to UC patients, where Th2 responses appear more prevalent. Pregnancy modulates these disease-underlying mechanisms to a different extent at different timepoints during gestation, which may further explain why disease modification is not always apparent. Nevertheless, several conclusions may be inferred from our current understanding of pregnancy-induced physiological changes. Overall, a beneficial effect of pregnancy on epithelial barrier function seems apparent, with relatively small fluctuations of pregnancy hormones already affecting the gut barrier. Furthermore, an overall image of induction of tolerance and suppression of immune responses during gestation is arising. With a predominant shift towards a Th2 phenotype, many reviews have speculated that in particular Th1-mediated diseases such as RA and CD may benefit from these pregnancy-induced changes, while Th2-diseases (such as SLE and UC) might be negatively affected (6,153,154). HCG, estrogen and progesterone rise rapidly during pregnancy and have shown several anti-inflammatory

actions in animal models. While most of these changes would be compatible with improvement of IBD disease activity, it has also been demonstrated that pregnant patients with SLE have lower levels of estrogen and progesterone in the third trimester of pregnancy compared to healthy controls, suggesting that some patient groups may benefit less from rises in pregnancy hormones (155). This also might be the case in UC, but studies to support this hypothesis are lacking. Lastly, changes in the microbiome occurring during normal pregnancy do not appear to be beneficial to IBD patients, but again, it is unclear to what extent these changes are modulated by pregnancy hormones, and to what extent microbial alterations are present in pregnant IBD patients. Thus, immune regulation in both pregnancy and IBD are complicated, and not static. Whether or not IBD disease course is affected by pregnancy may depend on individual patient's characteristics, including ongoing disease activity prior to conception, their microbiome and hormone/diet-induced changes therein and genetic underlying risk factors. Predicting which patients may experience reduced disease burden or increased disease activity during pregnancy and post-partum requires a better insight into the physiology and pathology of pregnancy and IBD.

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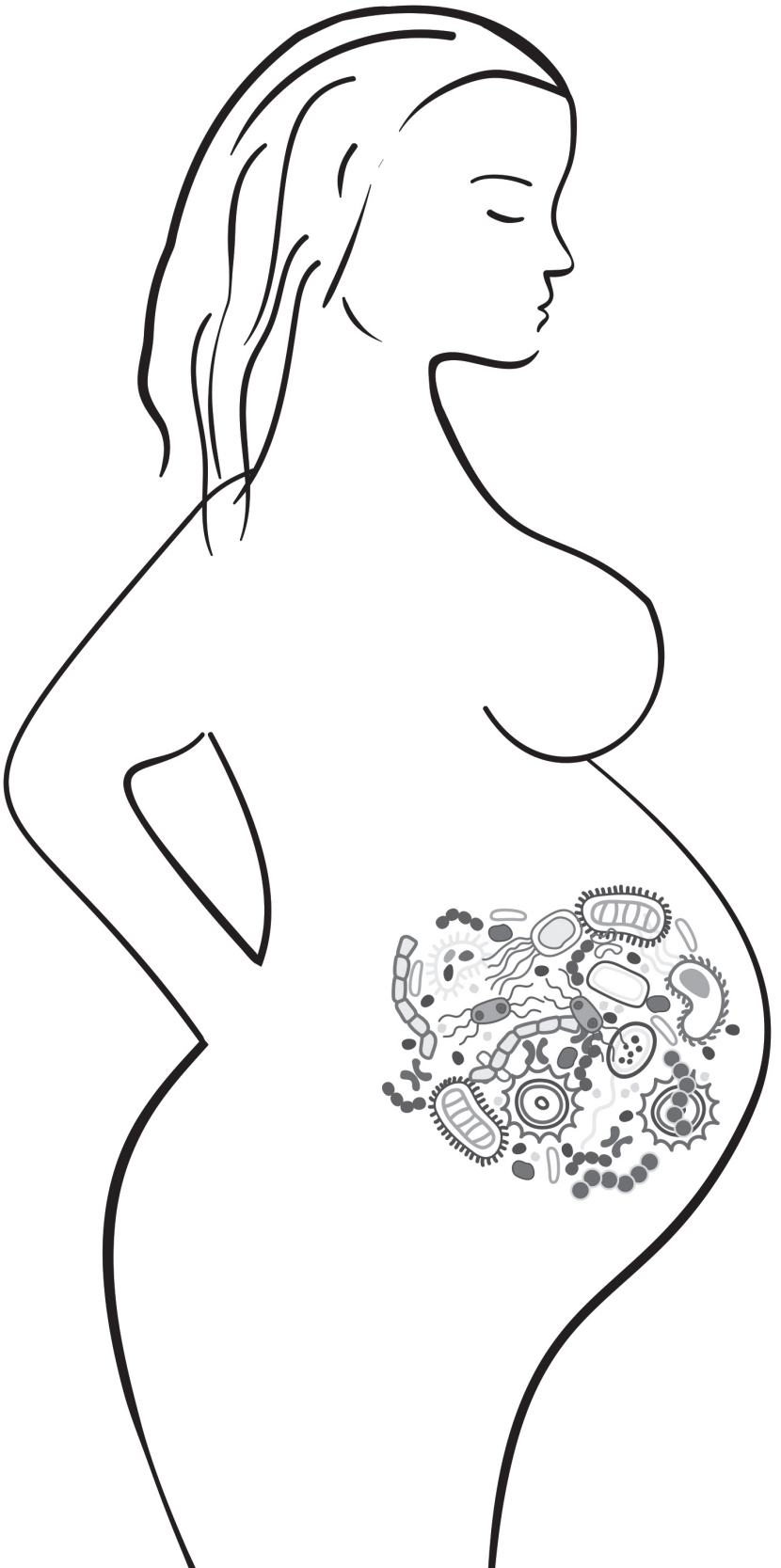

CHAPTER 3

A direct effect of sex hormones on epithelial barrier function in inflammatory bowel disease models

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ABSTRACT

Background Pregnancy is often described as an immune-tolerant state, and a disease modulatory role for pregnancy on IBD has been suggested. The direct effect of estrogen and progesterone on the intestinal epithelial barrier is underexplored. We therefore investigated the direct consequences of these pregnancy hormones on barrier cells and their function.

Methods We used IBD patient-derived inflammatory organoid models and 2D cell lines models. Epithelial barrier function was analyzed by measuring transepithelial electrical resistance, wound closure was determined by scratch assay, and cell viability was measured by MTT assays. Pro-inflammatory cytokine production was determined by enzyme-linked immunosorbent assays. Molecular modulation of endoplasmic reticulum (ER) stress induced by tunicamycin was studied by Western blot analysis of the ER stress markers GRP78, CHOP and p-IRE1.

Results Progesterone and estrogen improved wound healing and epithelial barrier function in intestinal epithelial cells via upregulation of tight junction proteins. Furthermore, these sexhormones significantly reduced ER-stress and reduce pro-inflammatory cytokine production in intestinal epithelial models.

Conclusion Our study shows that estrogen and progesterone alleviate ER stress, decrease pro-inflammatory cytokine production, stimulate wound healing, and increase barrier function of epithelial cells. Combined, these data suggest that pregnancy hormones can have beneficial effects on disease activity by positively modulating the intestinal epithelial lining.

INTRODUCTION

Inflammatory bowel disease (IBD), consisting of Crohn's disease (CD) and ulcerative colitis (UC), is a chronic debilitating disease. With a mean incidence peak between 15 and 25 years, patients are affected in their reproductive years (1,2), and concerns regarding pregnancy are therefore common in this patient group. Pregnancy is accompanied by hormonal, immunological and microbial changes, allowing the growth of an allogeneic fetus. In light of these physiological changes, a disease modulatory role for pregnancy has been speculated upon, and the effect of pregnancy on the course of IBD has been a topic of research for many years. However, studies have shown contradicting data. *Castiglione et al.* reported fewer disease relapses in the three years post-partum than before pregnancy in both CD and UC patients (3), suggesting a positive effect of pregnancy on disease parameters. A 10-year follow-up study confirmed this in both UC (from 0.34 to 0.18 relapses/year) and CD patients (from 0.76 to 0.12 relapses/year) (4). In addition, it appears safe to stop anti-TNFα treatment in pregnant IBD patients without increasing the risk of relapses (5,6). In contrast, *Pedersen et al.* reported a higher relapse rate during pregnancy (RR 2.19) and postpartum (RR 6.22) in pregnant UC patients than in non-pregnant UC patients, whereas the relapse rates were similar for pregnant and non-pregnant CD patients (7).

During pregnancy, the sexhormones estrogen and progesterone levels steadily increase until the 3rd trimester. Several studies have suggested that estrogen and progesterone have anti-inflammatory immune-modulatory effects by reducing inflammatory cytokines (8–10). Nevertheless, the direct effect of pregnancy hormones on IBD remains unclear. *Khalili et al.* showed that postmenopausal women who use oral contraceptives have a higher risk of developing mainly UC (11) which was further supported in a meta-analysis showing that the risk of developing CD increased with exposure to oral contraceptives, but normalized when contraceptives were stopped (12). In contrast, *Kane et al.* described a protective effect of estrogen on the bowel in women with IBD (13). Animal studies on the effect of pregnancy hormones on IBD have also shown conflicting data. HLA-B27 transgenic rats with chronic diarrhea had better stool scores after treatment with 17α-ethynyl-17β-estradiol for 5 days (14). Another animal study found an anti-inflammatory effect of a supraphysiological dose of 17β-estradiol in dextran sodium sulfate (DSS) murine model for colitis but a pro-inflammatory effect in dinitrobenzene sulfonic acid (DNB) colitis model (15), suggesting that the effect of estradiol on bowel symptoms may be context dependent. In rats with trinitrobenzene sulphonic acid (TNBS)-induced colitis, progesterone also significantly decreased oxidative damage in the colonic mucosa (16).

While immunological parameters have been extensively studied in the context of pregnancy, an underexplored avenue of investigation is the direct effect of estrogen and progesterone on the epithelial barrier. This barrier is the first line defense of the intestine against invading bacteria. In IBD patients, the epithelial lining is weakened and therefore less resistant to pathogens. This 'leaky gut' process is mainly caused by immune cells pro-

ducing inflammatory cytokines, such as IFNɣ an TNFα (17,18) and is worsened during active disease, when there is an additional reduction in tight junctions, which regulate the epithelial permeability (19). Furthermore, inflammatory triggers, such as cytokines and nitric oxide, can impede protein folding in IBD. This can lead to accumulation of unfolded proteins inside the endoplasmic reticulum (ER), which results in an unfolded protein response and ER stress. The gut epithelium expresses receptors for both estrogen (ERα and β) and progesterone (20) and animal studies have shown an increase in the transmembrane protein occludin in response to estrogen leading to improved intestinal epithelial barrier function (21). Moreover, the administration of estrogen or blockage of testosterone in male rats resulted in gut epithelial cells being more resistant to inflammation (22). Thus, while there is some evidence suggesting a modulatory role of sexhormones on intestinal barrier functions, it is as yet unclear whether these hormones, levels of which increased during pregnancy, directly affect epithelial barrier cells. Here, we employed human colonic adenocarcinoma cell lines (Caco2, HCT116, T84) and organoids from human colon biopsies as model systems to study epithelial barrier function, production of cytokines and ER stress modulation in response to sexhormones.

METHODS

Cell lines

Colorectal epithelial cell lines Caco2, HCT116 and T84 were cultured in Dulbecco's Modified Eagles Medium (DMEM, Lonza, Basel, Switzerland) supplemented with 100 U/mL penicillin, 100 mg/mL streptomycin (Life technologies, Bleiswijk, NL) and 10% Fecal Calf Serum (FCS, Sigma-Aldrich, St. Louis, USA). Cells were maintained at 37°C in a 5% CO_2 humidified setting.

Organoid culture

Non-inflamed intestinal biopsies were collected from three female UC patients undergoing endoscopy for evaluation of their disease. In two of these patients, no inflammation was observed, and organoids were generated from non-inflamed colonic biopsies. A third patient demonstrated endoscopic disease activity in the colon, and biopsies obtained from the lesion as well as biopsies obtained in a non-inflamed section of the colon were obtained for a paired comparison. Organoids were obtained as described (23). Biopsies were collected in PBS and transferred into a 15 mL tube containing 10 mL complete chelating solution (CCS, MilliQ H_2O was supplemented with 1.0 g/L of Na2HPO4-2H2O, 1.08 g/L of KH2PO4, 5.6 g/L of NaCl, 0.12 g/L of KCl, 15 g/L of Sucrose, 10 g/L of D-Sorbitol and 80 lg/L of DL-dithiothreitol). Crypts were isolated by adding 100 uL 0.5M EDTA for 35 minutes at 4°C followed by mechanical disruption of biopsies. Supernatant with crypts was treated with FCS and crypts were re-suspended in 12 mL cold advanced DMEM (Advanced DMEM/F12, 5 mL 100x GlutaMAX (GMX), 1% P/S, 500 µL Gentamicin and 5mL 1M HEPES). After

centrifugation, crypts were suspended in 50 µL growth factor reduced phenol-red free Matrigel (Corning, Bedford, USA). Then, a 50 µL droplet of Matrigel/crypt mix was placed in the center of each well of a 24-well plate, and was subsequently incubated at 37°C with 5% CO2 for 15 min. 700 µL of culture medium was added per well. The culture medium was supplemented with CMGF-, 2% of B-27 supplements (Gibco, Grand Island, USA), 1% of N2 Supplements (Gibco, Grand Island, USA), 500 pg/L of EGF, 1 mM n-Acetyl Cysteine, 10 mM Nicotinamide, 0.5 µM A83-01 (TGF-b inhibitor), 3 µM SB202190 (p38 inhibitor), 20% (vol/vol) of R-Spondin 1 (conditioned medium), 10% (vol/vol) of Noggin (conditioned medium) and 50% (vol/vol) of Wnt3a (conditioned medium). Culture medium was refreshed every 3 days, and organoids were passaged every 6-7 days. Each well contained 10 or more organoids.

Reverse transcriptase Polymerase chain reaction (rt-PCR)

We used rt-PCR to validate estrogen-beta and progesterone receptor expression on the intestinal cell lines, Ribosomal protein (*RP2*) primers were used as control (24). RNA was isolated using a NucleoSpin® RNA kit (MACHEREY-NAGEL, Düren, Germany) and cDNA was synthesized using the TAKARA reverse transcription system (TAKARA BIO INC, Shiga, Japan). PCR was performed in a 25µL reaction, using GoTaq polymerase and GoTaq Flexi buffer, 2 mM $MgCl_2$ (Promega, Madison, WI), dNTP (0.5mM each, Roche), 50 ng template and 0.5nM primer (for primers sequences see supplementary Table 1). Quantitative PCR (QPCR) was used to determine *claudin 1*, *claudin 2*, *zonula occludens 1* (*ZO-1*) and *occludin* expression. *HPRT1* primers were used as control. Per sample 10uL SYBR™ Select Master Mix and 0.5nM primer was used. All experiments were performed a minimum of 3 times.

Scratch assay

Scratch assays were performed on Caco2 and HCT116 cell lines as described (25). In short, cell monolayers were scratched with a pipette tip, washed twice, and treated with 1 µM estrogen and/or progesterone. Photographs were taken (Axiovert200 M microscope; Carl Zeiss BV, Sliedrecht, The Netherlands) to analyze the percentage of open wound area at 24 h (ImageJ software; US National Institutes of Health, Bethesda, MD, USA). Five independent wells were analysed per condition, with two measure-sites per scratch.

MTT

Cell viability was assessed using MTT assays as described (26). Cells were treated with estrogen and/or progesterone (Sigma Aldrich, St Louis, MA). After 24h, 48h and 72h, cells were incubated with 5mM MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma Aldrich, St Louis, MA) for 3h and colorimetric changes were measured using a microplate reader (Model 680XR Bio-Rad) at 490 and 595 nm. A minimum of three independent experiments were performed with each measurement in performed in duplicate.

TEER

Transepithelial resistance was measured using the Epithelial Voltohmmeter (EVOM2, Sarasota, FL, USA). Caco2 and T84 cells were seeded in a Transwell (6,5 mm insert, Costar, Kennebunk, USA) and grown to confluency. Cells were subsequently stimulated with 10 µM estrogen and/or progesterone and resistance was measured at 0, 24 and 72 hours. A minimum of three independent measurements were performed for time point.

Enzyme linked immunosorbent assay (ELISA)

Caco2 cells were plated at 900.000 per well in 24 wells plates. Upon attachment to the plate, cells were treated as described in the text and supernatant was harvested after 24h. Experiments with cells were performed four times and experiments with organoids were performed nine times. Cytokine levels in supernatants from intestinal cells and organoids were determined by ELISA (Ready-SET-Go!® eBioscience, San Diego, CA) as per manufacturer's instructions. All samples were tested in duplicate in the ELISAs.

Western blotting

Caco2, HCT116 cells and organoids were treated with tunicamycin (0.5µM) in the presence or absence of 10 µM estrogen and/or progesterone. Cells were lysed in Laemmli buffer (100mM Tris–HCl (pH 6.8), 200mM dithiothreitol, 4% SDS, 0.1% bromophenol blue, 20% glycerol, and 2% DTT) and proteins were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Merck chemicals BV, Amsterdam, the Netherlands) as described (27). Membranes were blocked in 50% odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE) in PBS/0.05% Tween-20 and incubated overnight at 4°C with primary antibody. After washing in PBS-Tween, membranes were incubated with IRDye® antibodies (LI-COR Biosciences, Lincoln, NE) for 1h. Detection was performed using Odyssey reader and analyzed using manufacturers software. All experiments were performed a minimum of three times.

Statistical analysis

For *in vitro* and *ex vivo* experiments, normality of distribution was assessed with D'agostino and Pearson Omnibus normality test. When passing normality test or when there were insufficient numbers to calculate normality, parametric testing was performed, otherwise, non-parametric tests were employed. Student T-tests were performed for comparisons of two groups. Mann-Whitney test for non-parametric data. For all tests, one or two-sided (as appropriate), p-values <0.05 were considered statistically significant, graphs show mean ± SEM or median with IQR. Analyses were performed using Graphpad Prism version 5.01.

RESULTS

Estrogen and progesterone stimulate wound healing in intestinal epithelium

One of the treatment goals in IBD is achievement of mucosal healing, as this is associated with reduced relapse rates and better quality of life (28). We therefore first investigated the effect of sexhormones on wound healing of epithelial barriers. After confirming the expression of progesterone and estrogen receptor in epithelial cells on mRNA level (supplementary Figure 1), 2D layers of Caco2 and HCT116 cells were scratched and the wound closure was measured in the presence or absence of progesterone and/or estrogen. Both the size of the wound, and the migrated distance of the wound-edges were determined. We found that when treated with both estrogen and progesterone, Caco2 cells showed a faster reduction in wound size than unstimulated cells ($p=0.006$ at $t=24$, Figure 1A), resulting in complete wound closure after 24h. In the less motile HCT116 cell line, complete wound healing was not achieved within this timeframe, but there was a faster migration of cells when stimulated with sexhormones ($p=0.044$ at $t=24$, Figure 1B). As a faster closure of wound area could conceivably also be achieved by increased proliferation, we next investigated the number of viable cells in the absence and presence of sexhormones. Figure 1C shows that neither progesterone nor estrogen or the combination thereof, substantially affects Caco2 or HCT116 cell growth. Together, these findings imply a positive modulatory role for sexhormones on epithelial wound healing.

Estrogen and progesterone alleviate ER stress in intestinal epithelium

Next, we wondered whether sexhormones may affect ER stress in intestinal barrier cells. We obtained inflamed and non-inflamed intestinal tissue biopsies from one UC patient in order to compare these two biopsies but did not observe differences in the IL-8 cytokine production, nor did we find significant differences in *claudin 1, claudin 2, occludin* or *ZO*-1 expression (supplementary Figure 2A-D). These data are in line with those of others, showing a loss of the inflammatory signature in *ex vivo* cultured organoids (29).To this end, we mimicked inflammation-induced cellular protein folding defects by stimulating human colonic organoids with tunicamycin, which resulted in upregulation of the ER stress markers GRP78 (BiP) and its downstream target CHOP, as well as phosphorylation of IRE1, a kinase which autophosphorylates upon cellular ER stress (30) (Figure 2). Thus, tunicamycin stimulation of organoids can serve as a model for epithelial barrier stress. Upon addition of sexhormones, ER stress induction as determined by GRP78 expression was attenuated, in particular in the case of progesterone (1.819 ± 0.4293 to 0.6333 ± 0.0333, $p=0.0420$, Figure 2A). CHOP expression was also reduced upon co-treatment of tunicamycin-treated organoids with either estrogen alone (2.062 ± 0.4497 to 0.8741 ± 0.1292, $p=0.0147$) or in combination with progesterone (2.062 ± 0.4497 to 0.9250 ± 0.08539, $p=0.0397$, Figure 2B). Lastly, we investigated IRE1, and showed that in our organoid model IRE-1 phosphorylation levels significantly decreased when estrogen (1.780 ± 0.2615 to 0.8600 ± 0.1208, $p=0.0127$),

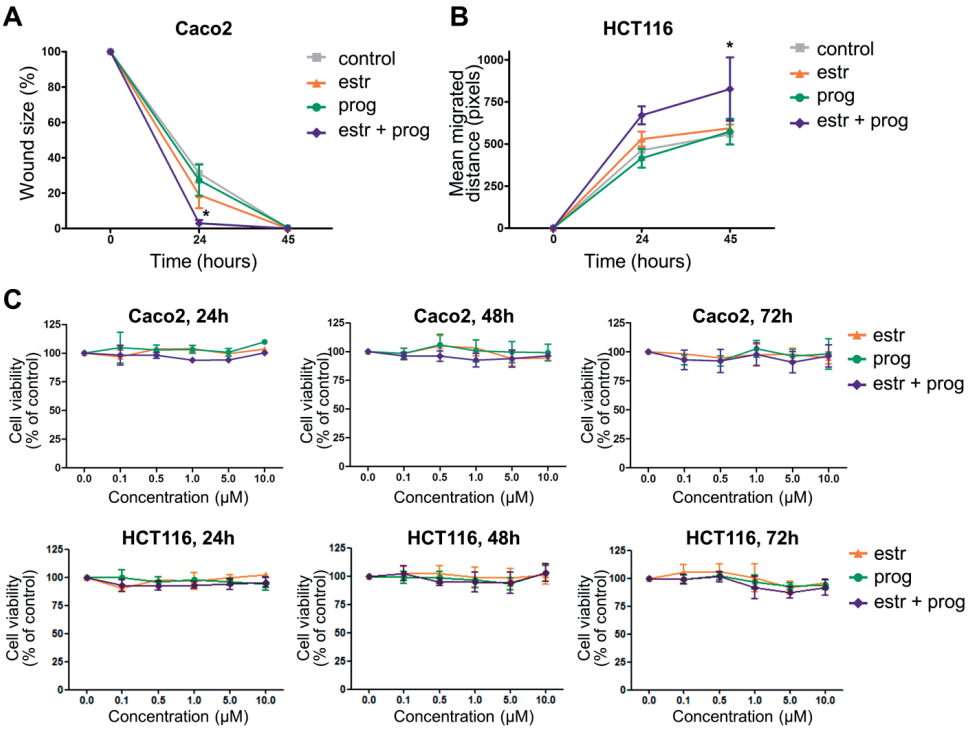

Figure 1. Estrogen and progesterone improve epithelial barrier healing. Caco2 cells (A) and HCT116 cells (B) confluent layers were scratched and cultured in the absence or presence of estrogen and/or progesterone. Cells were photographed at 0, 24 and 45 hours. Scratch assay of CACO2 cells is presented as mean percentage wound size. Scratch assay of HCT116 is presented as mean migration of wound edges in pixels. Results of five independent experiments are shown, with a minimum of two scratches per stimulus. (C) MTT assay shows that sexhormones do not affect viable cell numbers (concentrations of hormone treatment indicated on the X axis). Measurements were done in three independent experiments at t=24h, 48h and 72h.

progesterone (1.780 ± 0.2615 to 0.8200 ± 0.1020, p=0.0091) or the combination thereof were added (1.780 ± 0.2615 to 0.9400 ± 0.1631, p=0.0260, Figure 2C). We validated the stress relieving function of the sexhormones in the epithelial barrier models HCT116 and Caco2, again demonstrating that tumicamycin-induced GRP78 levels (supplementary Figure 3A,B) and IRE1 phosphorylation (supplementary Figure 3C) are reduced upon treatment of epithelial cells with progesterone, estrogen or the combination thereof. Thus, the data demonstrate that sexhormones may protect epithelial barrier cells from damage inflicted by ER stress.

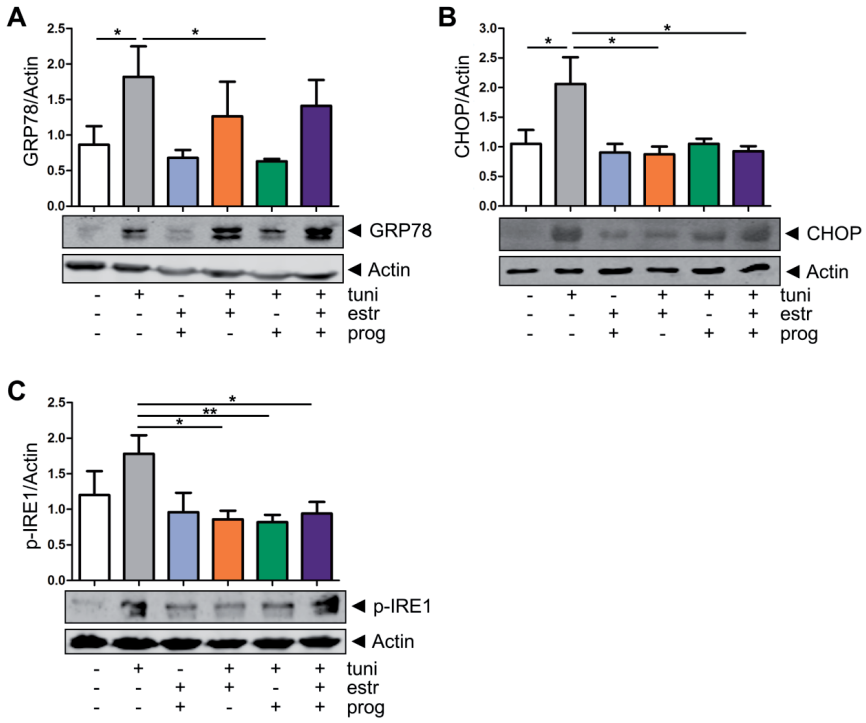

Figure 2. Sexhormones reduce ER stress in IBD organoids. Western blot analysis of the ER stress markers GRP78 (A) CHOP (B) and p-IRE1 (C) in organoids. Induction of ER stress by stimulation with tunicamycin for 20 hours results in an upregulation GRP78 protein expression. Four hours of stimulation with 10nM tunicamycin resulted in an upregulation of CHOP protein expression and 6 hour of stimulation with 10nM tunicamycin resulted in an upregulation of the p-IRE1 protein expression. Addition of estrogen or progesterone attenuates ER stress induction. Upper panels show mean densitometry values of the ER stress proteins, corrected for actin levels in the same lanes, of five independent experiments, lower panels show representative examples of the blots.

Pro-inflammatory cytokine production by intestinal epithelial cells is decreased in the presence of progesterone and estrogen

Deregulation of pro-and anti-inflammatory cytokines and interleukins is seen in IBD. In particular IL8, a neutrophil chemoattractant, and IL6, involved in perpetuating the immune reaction, are found in increased quantities in inflamed mucosa (31). We therefor investigated these cytokines in our cell culture models. Excretion of IL8 and IL6 were low in resting organoids and Caco2 cells, and thus not modulated by progesterone and estrogen (Figure 3A-C). However, treatment of cells with tunicamycin enhanced the levels of these pro-inflammatory cytokines, further demonstrating the pro-inflammatory effect of ER stress induction in these model systems. Next, we tested whether estrogen and progesterone influence this stress-induced cytokine production by organoids and Caco2 cells.

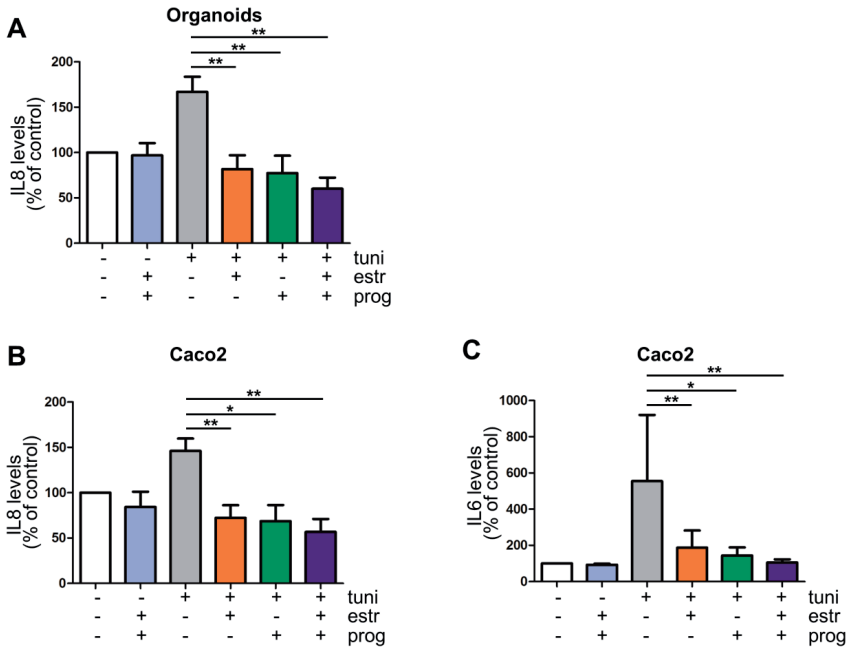

Figure 3. Progesterone and estrogen reduce IL6 and IL8 secretion in IBD inflammation models. Estrogen and progesterone significantly reduce IL8 production in an inflammation organoid model of IBD (A, n=9) as well as the Caco2 cell line model (B, n=4). IL6 production in Caco2 cells was also decreased by sexhormones (C, n=4).

As shown in Figure 3A, IL8 production in tunicamycin-treated organoids was significantly reduced when stimulated with estrogen, progesterone and both (p=0.0040 vs p=0.0078 vs p=0.0040 respectively, Figure 3A), which was also seen in Caco2 cells (p=0.0092 vs p=0.0138 vs p=0.0040 respectively, Figure 3B). Furthermore, when Caco2 cells co-stimulated with estrogen, progesterone and both sexhormones a decrease of IL6 cytokine was measured (p=0.0093 vs p=0.0125 vs p=0.0021 respectively, Figure 3C). IL6 was not detected in organoids cultures, nor were TNFα or IL10.

Improved barrier function strength in the presence of estrogen and progesterone

In IBD, weakening of the intestinal lining can result in a decreased resistance to pathogens. For the determination of the integrity of this epithelial barrier we measured the transepithelial electrical resistance (TEER). Compared to controls, in Caco2 cells the barrier function was increased by estrogen (6007 ± 105 to 8347 ± 140, p=0.0002, t=72h, Figure 4A) as well as the combination of progesterone and estrogen (6007 ± 105 to 8177 ± 180, p=0.0005, t=72 h, Figure 4A), although this latter combination treatment was not more efficient as compared to estrogen alone. As the growth pattern of HCT116 cells does not result in a confluent cell layer allowing TEER measurements, we further validated these results in another

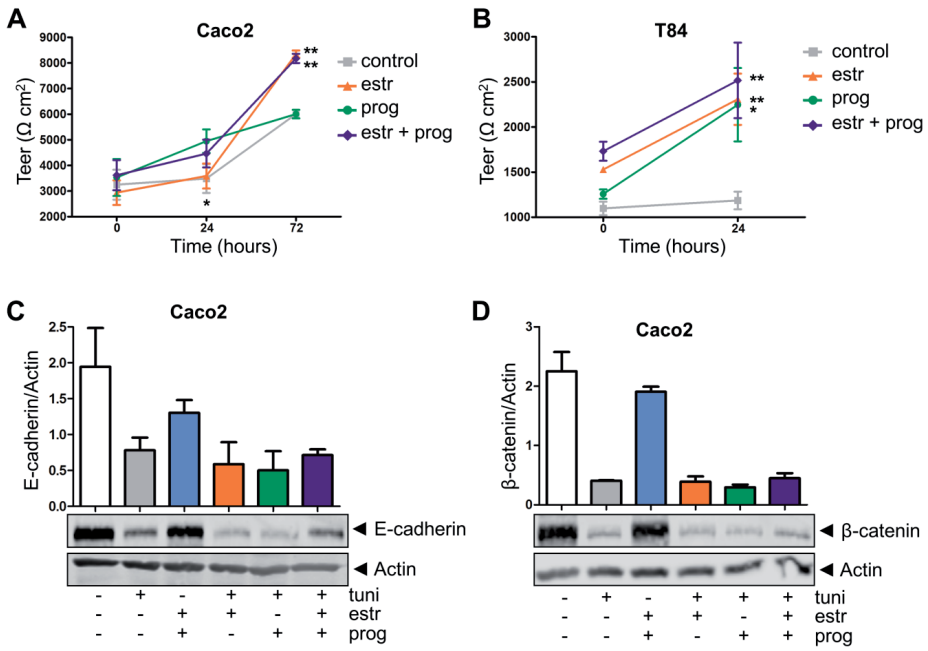

Figure 4. Sexhormones improve epithelial barrier function. TEER measurement of Caco2 (A) and T84 cell lines (B) show an increase in resistance when stimulated with sexhormones. Results of five independent experiments are shown. (C-D) Western blot analysis of the adhesion junction molecules E-cadherin (C) and β-Catenin (D). Neither constitutive levels nor stress-induced decreases in E-cadherin or β-Catenin levels are modulated by sexhormones. Upper panels show densitometry values (corrected for actin levels in the same lane) of two independent experiments, lower panels show representative examples.

intestinal epithelial cell line, T84, which does form a resistant monolayer. As for Caco2 cells, barrier function was improved in T84 cells stimulated with sexhormones for 24 hours. Both estrogen and progesterone alone increased TEER in these cell layers (neg vs estr 1186 ± 97 to 2307 ± 284, p=0.0022; neg vs prog 1186 ± 97 to 2247 ± 407, p =0.0195), as did the combination treatment also improved barrier strength (neg vs estr+prog, 1186 ± 97 to 2516 ± 418, p=0.0045, Figure 4B).

In an effort to clarify the molecular mechanisms contributing to improved barrier function upon sexhormone treatment, we first determined protein levels of E-cadherin and β-catenin, two adherent junction proteins modulating epithelial cell-cell adhesion (32). However, treatment with a combination of progesterone and estrogen did not modulate expression levels of these proteins (see Figure 4C,D for Caco2 cells, supplementary Figure 5A,B for HCT116 cells). As a control, we showed that stress induction in these epithelial cells with tunicamycin reduced the expression of E-cadherin or β-catenin, but again, this was not modulated by sexhormones (Figures 4C,D, supplementary Figure 5A,B). Next, we investigated whether an improvement in tight junction dynamics could underlie the positive

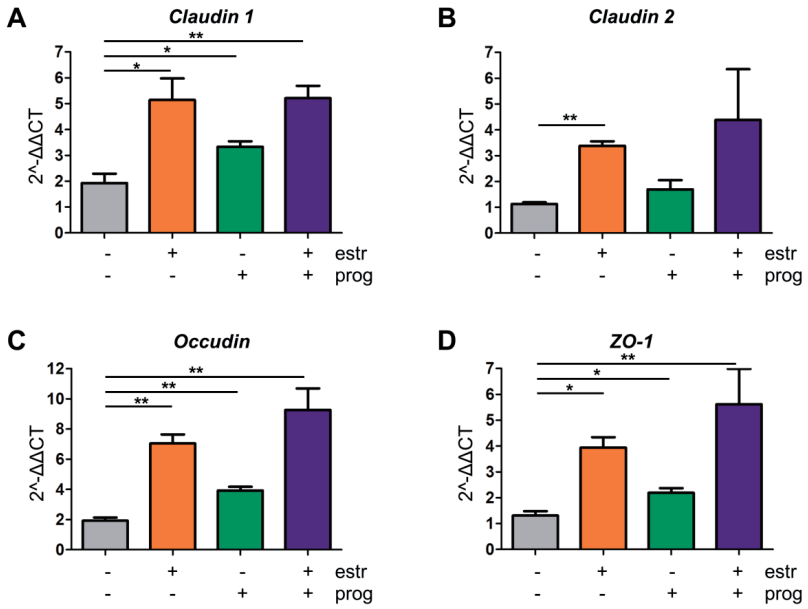

Figure 5. Improvement of tight junction dynamics by sexhormones. QPCR analysis of the tight junction claudin 1 (A), claudin 2 (B), ZO-1 (C) and occludin (D) were measured in the IBD organoid inflammation model. Co-stimulation with estrogen, progesteron or a combination resulted in an upregulation of claudin 1, ZO-1 and occludin (three independent experiments). For claudin 2 this was only for co-stimulation with estrogen (five independent experiments).

effect of the sexhormones on barrier integrity. QPCR analysis showed that in our organoid inflammation model, *claudin 1* expression was significantly upregulated upon treatment with either estrogen, progesterone or both (p=0.0239, p=0.0280 and p=0.0053, respectively, Figure 5A). Similarly, mRNA levels of and *occludin* (p=0.0013, p=0.0034 and p=0.0069, respectively, Figure 5C) and *ZO-1* (p=0.0040, p=0.0242, p=0.0359, respectively, Figure 5D) increased upon treatment with either sexhormone alone or the combination thereof. For *claudin 2* significant modulation was only observed with estrogen alone (p=< 0.0001, Figure 5B). Caco2 and HCT116 cells showed a similar tendency to upregulate *claudin 2* and *ZO-1* upon sexhormone treatment (supplementary Figure A,B). Thus, while estrogen and progesterone positively modulate the barrier lining, the molecular mechanisms involved do not appear to include the E-cadherin/ β-catenin complex, but affects tight junction dynamics.

DISCUSSION

Several (auto)immune diseases, most noticeably rheumatoid arthritis and multiple sclerosis, have been shown to improve during pregnancy, a phenomenon that is generally ascribed to modulation of immunological parameters in response to rising progesterone and estrogen

levels (33–35). However, pregnancy can induce a range of physiological changes, including modulation of the gastrointestinal microbiome as well modulation of the gastrointestinal smooth musculature (36). The complex disease etiology of IBD involves an altered immune response towards the intestinal microbiome in genetically susceptible individuals. Thus, with all these changes during pregnancy occurring in processes that affect IBD pathophysiology, a beneficial effect of pregnancy in IBD has been speculated upon (37). An important aspect of IBD pathophysiology includes a weakened intestinal barrier function, yet the direct consequences of pregnancy hormones on intestinal barrier cells thus far remained unclear. Here, we show that progesterone and estrogen can directly improve epithelial barrier functions, suggesting a positive modulatory effect of these hormones on the intestinal epithelial lining during pregnancy.

Firstly, our results showed that estrogen and progesterone, without affecting cell proliferation, improved wound healing and epithelial barrier strength. These results are in line with those of *Braniste et al.*, who concluded that there is a physiological link between estrous cycle-dependent changes in hormone levels and intestinal permeability changes, and also demonstrated an ERβ-mediated increase of *occludin* in epithelial cells in the colon (38). We now demonstrate that this also applies for *claudin 1*, *claudin 2* and *ZO-1*. Secondly, we show that estrogen and progesterone can alleviate ER stress in intestinal epithelial cells. We and others have previously suggested that mucosal ER stress is linked to the development of IBD (39–41). ER stress is known to induce inflammatory responses, and contributes to a rise in pro-inflammatory cytokines in several cell types (42). Here, we demonstrate that induction of ER stress also increases IL8 and IL6 levels in our epithelial models, including IBD organoids, and we thus employed induction of ER stress as a model of inflammation in these systems. Several groups have now cultured organoids from IBD patients, and these cultures provide a good model to investigate the barrier function in an IBD-specific context (43). However, while some patient and barrier-specific transcription patterns and functions are present in these *in vitro* cultures, other studies have also suggested that inflammation in these organoid systems is not propagated, and may be best modelled by addition of exogenous stimulants (44), as inflammatory phenotype might be lost upon culture of organoids beyond 4 weeks (29). Our data suggest that estrogen and progesterone may reduce inflammation-induced pro-inflammatory cytokine production by epithelial barrier cells. Both IL6 and IL8 Levels are increased in the inflamed mucosa from IBD patients (45,46), and our own observations suggest that pro-inflammatory cytokine levels (e.g. IL6, IL8, TNFα) decrease significantly in IBD patients upon conception (unpublished data). Our current study suggests that this might be partly due to a direct effect of pregnancy hormones on cytokine production in epithelial cells. A study showing significantly lower systemic plasma cytokine levels of IL6, IL8 and IL10 in premenopausal women than in men corroborates this notion (47).

To our knowledge, this is the first study reporting on the direct effect of pregnancy hormones on different aspects of the intestinal epithelial barrier. One limitation of this study is

that we employed one concentration of the sexhormones. During pregnancy both estrogen and progesterone increase, but hormone levels *in vitro* are not one-on-one comparable with hormone levels *in vivo*, and the modulation of levels of these steroid hormones in the mucosal barrier are as yet unknown. Thus, it is conceivable that we may have used supra physiological levels when adding 10 μM estrogen and progesterone as a stimulus *in vitro*. We used equimolar concentrations of the hormones for a fair comparison between these hormones. Our results imply that both progesterone and estrogen have beneficial effects on epithelial barrier functions, with additive effects on most processes studied here, which allows statistical significance to be reached while not reaching this significance with one or both of the single treatments. Another limitation of this study is that the effect of IBD medication is not taken into account in these *in vitro* models. However, our own unpublished data suggest that IBD medication does not affect cytokine production in pregnant IBD patients patients. 5-ASA and azathioprine are considered of low risk during pregnancy and therefore often used in clinical practice Studies investigating the effect of these treatments on the epithelial barrier function have indicated a positive effect in an intestinal organoids model (48). Furthermore, 5-ASA was able to inhibit IFN-gamma induced impairment of the epithelial barrier (49). Thus, overall the effect seems to be positive rather than negative and suggest that there might be an additional positive effect of some IBD medication on the epithelial barrier. Nevertheless, our study provides additional insight into the mechanism of action of estrogen and progesterone on the intestinal epithelial lining and support a protective role of pregnancy hormones on epithelial barrier function during pregnancy.

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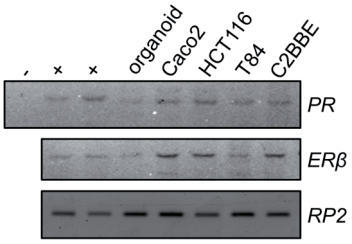
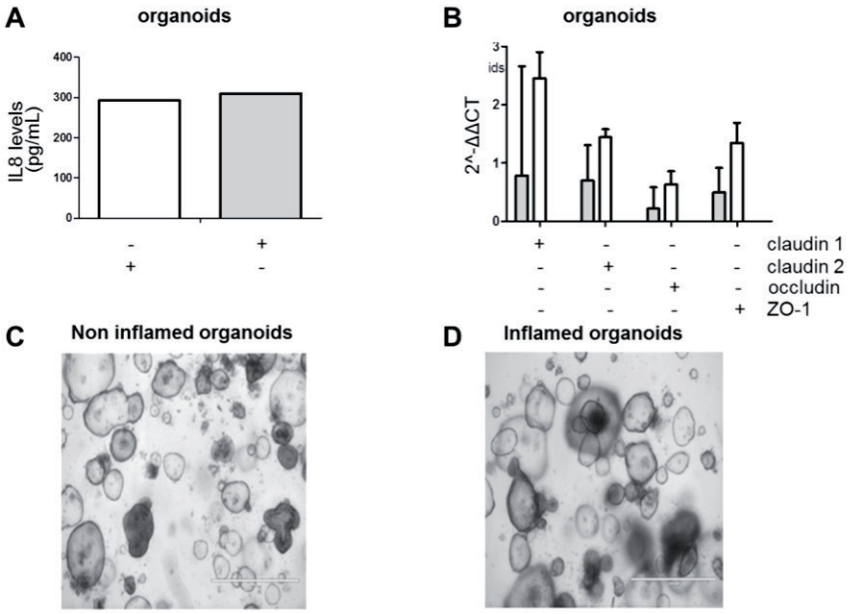
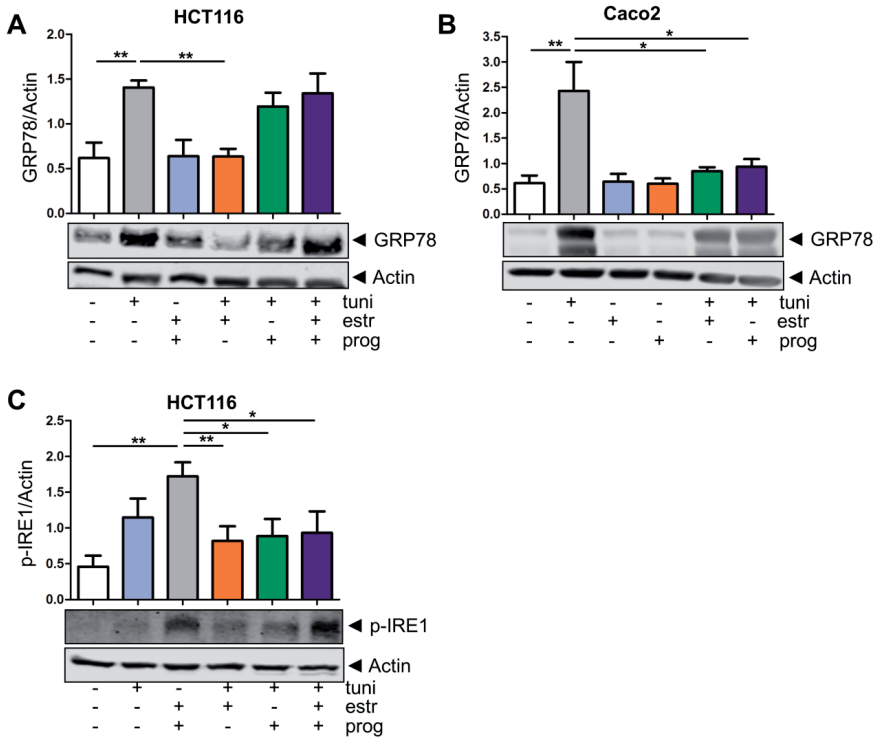
SUPPLEMENTARY MATERIALS



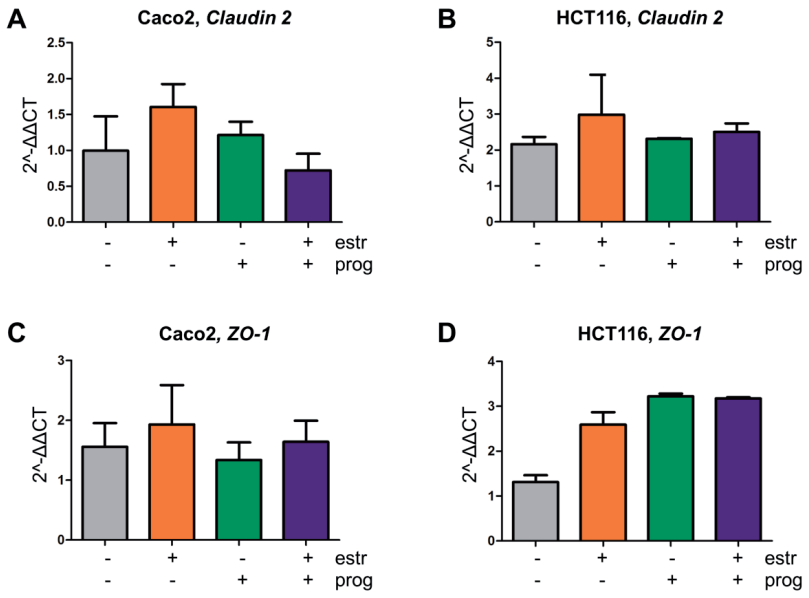
Supplementary Figure 1. Presence of progesterone (PR) and estrogen-beta (ERβ) receptors mRNA was tested by PCR. Ribosomal protein 2 (RP2) was used as cDNA quality control. mRNA extracted from placental tissue was used as positive control (+), water was used as negative control (-) for the PCR.



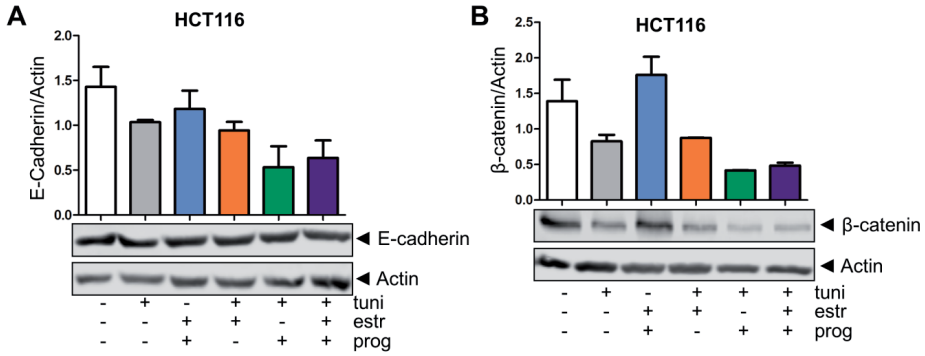
Supplementary Figure 2. ELISA analysis showed no differences in IL8 levels in inflamed vs non-inflamed organoids (A). QPCR analysis showed no differences in claudin 1, claudin 2, occludin and ZO-1 expression (B). Organoids derived from non-inflamed biopsies (C). Organoids derived from inflamed biopsies (D) were slower to grow, but did not show morphological differences.



Supplementary Figure 3. Sexhormones reduce ER stress in cell lines. Western blot analysis of the ER stress marker GRP78 in HCT116 (A, n=3) and Caco2 (B, n=6). Induction of ER stress by stimulation with tunicamycin for 20 hours results in an upregulation GRP78 protein expression, which was decreased upon treatment with estrogen in HCT116 cells and progesterone or the combination of sexhormones in Caco2 cells. Similarly, phosphorylation of IRE1 in HCT116 cells (C) was reduced upon treatment with sexhormones (n=5). Upper panels show mean densitometry values of the ER stress proteins, corrected for actin levels in the same lanes, lower panels show representative examples of the blots.



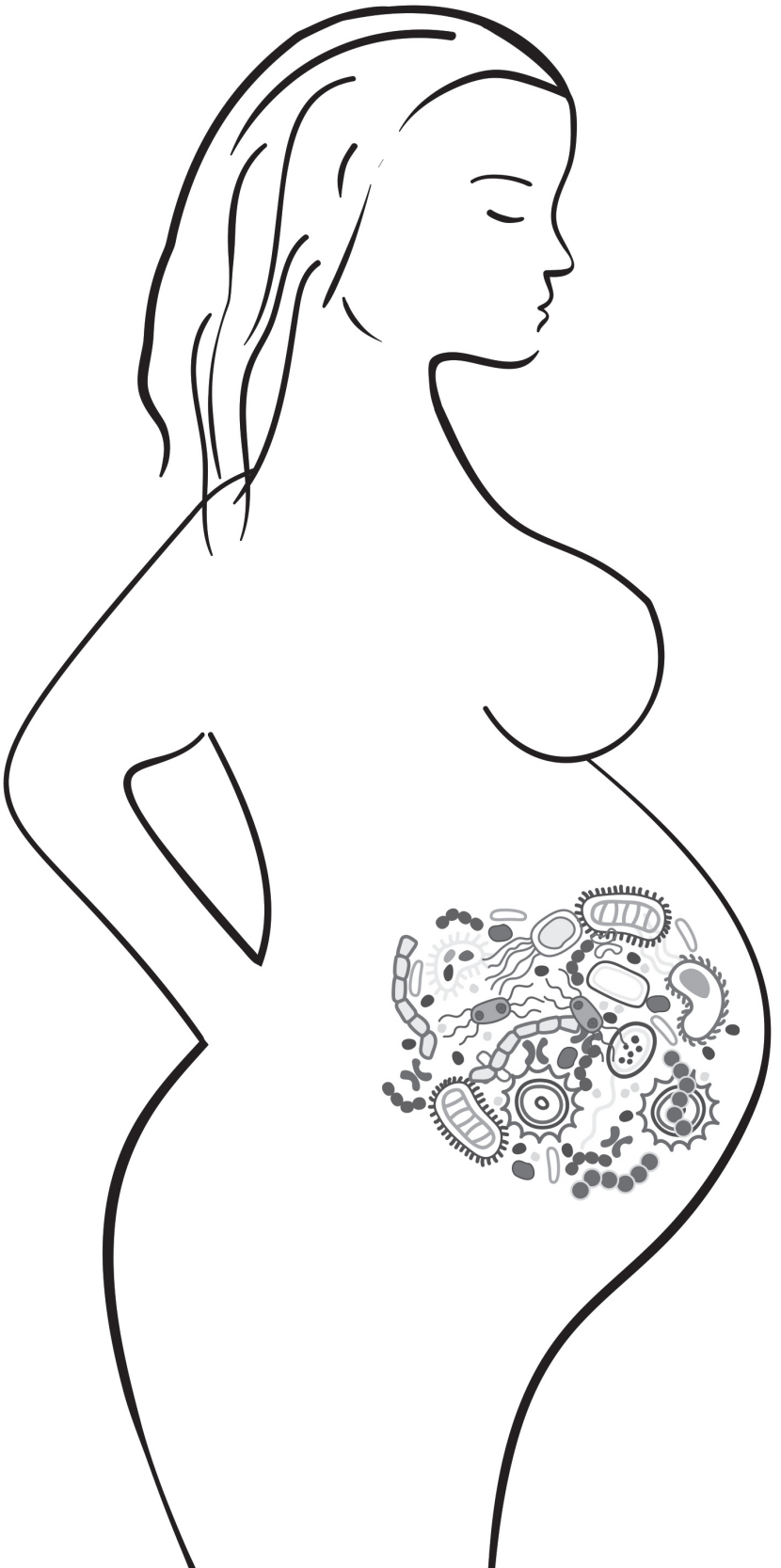
Supplementary Figure 4. Improvement of tight junction dynamics. QPCR analysis of the tight junction claudin 2 in Caco2 cells (A), claudin 2 in HCT116 cells (B), ZO-1 in Caco2 (C) and ZO-1 in HCT116 (D). For CACO2 the results of four experiments are shown, for HCT116 of two experiments.



Supplementary Figure 5. Neither constitutive levels nor stress-induced decreases in E-cadherin or β-Catenin levels are modulated by sexhormones in HCT116 cells. Upper panels show densitometry values (corrected for actin levels in the same lane) of two independent experiments, lower panels show representative examples.

Supplementary Table 1

Estrogen receptor	forward: 5'-TGAAAAGGAAGGTTAGTGGGAACC, Reverse: 5'-TGGTCAGGGACATCATCATGG
Progesteron receptor	forward: 5'-GATTCAGAAGCCAGCCAGAG, Reverse: 5'-TGCCTCTCGCCTAGTTGATT
Claudin 1	forward: 5'-TGGTGGTTGGCATCCTCCTG-, Reverse: 5'-AATTCGTACCTGGCATTGACTGG
Claudin 2	forward: 5'-GGCGGTAGCAGGTGGAGTC, Reverse: 5'-CTTGGTAGGCATCGTAGTAGTTGG
ZO-1	forward: 5'-CAAGATAGTTTGGCAGCAAGAGATG, Reverse: 5'-ATCAGGGACATTCAATAGCGTAGC
Occludin	forward: 5'-AATTCTTCACTTCTAACAAATGGACCTC, Reverse: 5'-CACATCACAATAATGAGCATAGACAGG
Ribosomal protein (RP2)	Forward: 5'-AAGCTGAGGATGCTCAAAGG, Reverse: 5'-CCCATTAAACTCCAAGGCAA
HPRT1	forward: 5'-TGACACTGGCAAAACAATGCA, Reverse: 5'-GGTCCTTTTCACCAGCAAGCT



CHAPTER 4

Modulation of cytokine patterns and microbiome during pregnancy in IBD

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ABSTRACT

Objective Pregnancy may affect the disease course of IBD. Both pregnancy and IBD are associated with altered immunology and intestinal microbiology. However, to what extent immunological and microbial profiles are affected by pregnancy in patients with IBD remains unclear.

Design Faecal and serum samples were collected from 46 IBD patients (31 Crohn's disease (CD) and 15 UC) and 179 healthy controls during first, second and third trimester of pregnancy, and pre pregnancy and postpartum for patients with IBD. Peripheral blood cytokine profiles were determined by ELISA, and microbiome analysis was performed by sequencing the V4 region of the bacterial 16S rRNA gene.

Results Proinflammatory serum cytokine levels in patients with IBD decrease significantly on conception. Reduced interleukin (IL)-10 and IL-5 levels but increased IL-8 and interferon (IFN)γ levels compared with healthy controls were seen throughout pregnancy, but cytokine patterns remained stable during gestation. Microbial diversity in pregnant patients with IBD was reduced compared with that in healthy women, and significant differences existed between patients with UC and CD in early pregnancy. However, these microbial differences were no longer present during middle and late pregnancy. Dynamic modelling showed considerable interaction between cytokine and microbial composition.

Conclusion Serum proinflammatory cytokine levels markedly improve on conception in pregnant patients with IBD, and intestinal microbiome diversity of patients with IBD normalises during middle and late pregnancy. We thus conclude that pregnancy is safe and even potentially beneficial for patients with IBD.

SIGNIFICANCE OF THIS STUDY

What is already known on this subject?

- Pregnancy constitutes a unique physiological state in which immune and microbial changes prepare the female body for fetal implantation, growth and nourishment.
- Immune dysfunction and microbial dysbiosis are common features in patients suffering from IBD.
- Some immune diseases show amelioration of disease activity during pregnancy suggesting that pregnancy-induced physiological changes affect disease pathology.
- For many patients with IBD, pregnancy is associated with uncertainties regarding disease activity and outcomes for the fetus.

What are the new findings?

- Proinflammatory cytokines (interleukin (IL)-6, IL-8, IL-12, IL-17 and tumour necrosis factor (TNF)α) decrease significantly on conception in patients with IBD.
- Healthy women show pregnancy-associated changes in serum cytokines during the trimesters of pregnancy that are not seen in pregnant patients with IBD.
- IBD immunological state as assessed by serum cytokine profiles are patient specific but not influenced by disease type, inflammation or pregnancy trimester.
- Both patients with UC and Crohn's disease display a disease manifestation-specific but low-diverse microbiome before and during early pregnancy.
- IBD-associated dysbiosis as assessed by microbial diversity disappears during middle and late pregnancy.
- Cytokine and microbial network and dynamic analysis provide detailed data on correlations between microbiome, disease type, cytokine profiles and pregnancy.

How might it impact on clinical practice in the foreseeable future?

- From an immunological and microbiological viewpoint, pregnancy in patients with IBD is beneficial and can be safely recommended to patients.
- As pregnancy is associated with changes of the intestinal microbiota, microbiome-directed interventions (eg, faecal transplantations, antibiotics or probiotic therapies) are not recommended in pregnancies complicated by IBD.

INTRODUCTION

IBD, including Crohn's disease (CD) and UC, are complex multifactorial diseases. Impaired epithelial barrier function, environmental triggers, genetic susceptibility, as well as an ineffective immune reaction towards the intestinal microbiota, contribute to a chronic intermittent intestinal inflammation (1,2). As IBD affects women in their reproductive years, a common concern for patients with IBD is how pregnancy will affect their disease course, and conversely, how the disease will affect their pregnancy and fetal health. These concerns are not unfounded, as active disease during conception and pregnancy has been associated with worse pregnancy outcomes (3,4), and children of patients with IBD are themselves at increased risk of developing IBD (5) due to genetic risk factors as well as parental environmental and microbial factors (6-9). Conversely, however, the effect of pregnancy on the maternal IBD disease course is less clear. A postpartum reduction of flares has been observed in both patients with CD and UC (10,11), although these data were disputed by Pedersen *et al. (12),* who found an increased risk of flares in patients with UC both during pregnancy and postpartum. Pregnancy constitutes a unique state, in which hormone-induced physiological changes prepare the body for implantation, fetal growth and parturition. Changes that take place include modulation of immune function to allow for development of a major histocompatibility complex (MHC)-mismatched fetus (13). Thus, maternal immune tolerance was long thought to be increased throughout pregnancy; however, it is now becoming clear that immunological states fluctuate during pregnancy to support different needs at its different stages. Successful implantation requires a pro inflammatory Th1 environment at the maternal–fetal interface, which is followed by a shift towards a more tolerogenic Th2 response for the main duration of pregnancy, with again increased polarisation to a Th1 response shortly before partition (14,15). However, it is unclear to what extent placental immunological shifts actually translate to systemic immunological changes capable of affecting disease activity in the intestinal tract, as surprisingly few studies have investigated peripheral changes across the different trimesters of pregnancy, and those that did show conflicting results (16-18).

In addition to immunological changes, it has been shown that the intestinal microbiome is altered during healthy pregnancy, with reduced microbial diversity observed in the third trimester, which was shown to be similar to the microbiome in patients with metabolic disease (19). While it is known that the microbiome and the immune system are in close reciprocal relationship (20-23), it is as yet unclear whether immunological and microbial changes during pregnancy are correlated. Third trimester stool microbiota was shown to harbour inflammatory characteristics, with an overall increase in Proteobacteria and Actinobacteria, and it is possible that such alterations contribute the inflammatory environment needed for parturition and prepare the maternal body for the energy demands imposed by lactation (24). In non-pregnant patients with IBD, alterations in microbial signatures are already present, with reduced faecal bacterial diversity, decreased presence of commensal

butyrate producing bacteria (eg, *Faecalibacterium prausnitzii*) and increased abundance of Proteobacteria and Actinobacteria reported as some of the most consistent findings (25-27). Whether pregnancy in patients with IBD further modulates the intestinal microbiota and whether microbial changes during pregnancy are associated with diseases state are currently unknown.

While pregnancy clearly affects many physiological processes that are deregulated in IBD, remarkably little is known about immune and microbial signatures in patients with IBD during pregnancy. Here, we compared peripheral blood cytokine patterns and faecal microbiome from pregnant patients with IBD and pregnant healthy controls and show that unlike healthy controls, pregnancy in IBD is not accompanied by major changes in peripheral cytokine patterns in our cohort. Furthermore, differences in microbial diversity that are present between patients with UC and CD, and IBD versus controls disappear during pregnancy.

MATERIALS AND METHODS

Patient recruitment

Women diagnosed with IBD visiting the preconception outpatient clinic at the Erasmus MC University Medical Center, Rotterdam, between March 2014 and June 2016 were asked to donate stool and serum in the first, second and third trimester (T1–T3), where possible samples were also obtained pre pregnancy and postpartum (NL47357.078.13 Dutch Medical Ethical Committee). Exclusion criteria included inability to provide consent. For patients with IBD, disease type, surgical history, age of diagnosis and age at inclusion were noted, and for each time point of sample collection, medication use, flaring of disease (as assessed by clinician based on clinical findings, faecal calprotectin and/or endoscopy) and disease activity score (Harvey Bradshaw index (HBI) for CD (28) and Simple Clinical Colitis Activity Index (SCCAI) for UC and IBD-unclassified (29) were noted. Healthy controls were recruited at Rabin Medical Center, Petah- Tikva, Israel (Institutional Review Board Approval number 0263-15-RMC and 0608-18-RMC) and in Clalit HMO clinics at Petah-Tikva district Israel (Approval number 0135-15-COM). Following recruitment, participants provided blood and faecal samples at T1, T2 and T3. All procedures used for collection were in accordance with National Institutes of Health Human Microbiome Project standards (30). All participants signed informed consent.

Sample preparation and sequencing

DNA was extracted from 0.25 g faeces of healthy pregnant women using the Power Soil DNA Isolation Kit (MoBio, Carlsbad, USA), according to the manufacturer's instructions and following a 2 min bead-beating step (Biospec, Bartlesville, Oklahoma, USA). From pregnant women with IBD, the bacterial DNA was extracted using PureLink Microbiome DNA

Purification Kit (Invitrogen, Carlsbad, California, USA) following a 2 min bead-beating step. The V4 region of the bacterial 16S rRNA gene was amplified from the extracted DNA using the 515F and 806R barcoded primers following the Earth Microbiome Project protocol (31). Each PCR reaction consisted of 2 µL 515F primer (10 µM), 2 µL 806R primer (10 µM), 25 µL prime star max PCR mix (Takara, Mountain View, California, USA), 17 µL ultra-pure water and 4 µL of sample DNA. DNA amplification consisted an initial denaturing step for 3 min at 95°C followed by 30 cycles of denaturation (98°C for 10 s), annealing (55°C for 5 s) and extension (72°C for 20 s), with a final elongation step at 72°C (for 1 min). Amplicons were purified using AMPure magnetic beads (Beckman Coulter, Brea, California, USA) and DNA concentration was quantified using Qubit dsDNA HS Assay (Thermo Fisher, Bartlesville, Oklahoma, USA). Samples were then pooled at equal concentrations (50 ng/µL) and purified again using 2% E-Gel (Invitrogen). DNA fragments of the appropriate size were purified using NucleoSpin Gel and PCR Clean-up (Macherey-Nagel, Düren, Germany) and sequenced using the Illumina MiSeq platform at the Genomic Ccenter, Azrieli Faculty of Medicine, Bar-Ilan University, Israel.

Microbiome analysis

Data analysis was performed using QIIME2 (32). Sequence reads were demultiplexed, and sequenced reads were error-corrected by Divisive Amplicon Denoising Algorithm (33). A phylogenetic tree was constructed and features were assigned taxonomy using Greengenes reference database (34). Alpha and beta diversity measures were calculated based on a feature table with samples containing at least 5928 sequences. Richness and evenness (alpha diversity parameters) were calculated using the Faith's Phylogenetic Diversity (35), Shannon's Diversity Index and Pielou's Evenness measures (36). For between sample diversity (beta diversity), weighted and unweighted UniFrac distances were calculated (37). Over-represented and under-represented features were identified using linear discriminant analysis effect size (LEfSe) (38).

Normalisation

Features were merged to the genus level by averaging over all features assigned to the same genus. Given the large variation in feature values, we transformed these values to Z scores by adding a minimal value to each feature level (0.01) and calculating the 10-basis log of each value. Statistical whitening was then performed on the table by removing the average and dividing by the SD of each feature. The average of each normalised bacteria over each time point was to remove the effect of time on the samples.

Machine learning

Unsupervised learning was performed on the normalised and merged version of the 16S rRNA feature table in order to recognise patterns in the data. Principal component analysis (PCA) was performed using Python version 3.5 and its package sklearn. A two-tailed

p value of less than 0.05 was considered statistically significant. A linear support vector machine was used to classify patients with UC from patients with CD using 40 support vectors. Leave-one-out cross-validation was performed. The box constraint value was 1. More complex methods were not used to limit overfitting, given the limited number of samples.

ELISA

Blood was collected in serum separator tubes (BD Bioscience, Mississauga, Ontario, Canada). Serum was aliquoted to avoid repeated freeze–thaw cycles and stored at −80°C until analysis. ELISA for the interleukins IL-4, IL-5, IL-6, IL-8, IL-9, IL-10, IL-12, IL-15, IL-17, IL-21, TNFα and IFNγ were performed using a kit according to manufacturer's protocol (Ready-SET-Go! eBioscience, San Diego, California, USA). When insufficient serum was available, a subset of cytokines was measured. PCA of cytokine data were visualised using ClustVis (39). Unit variance scaling was applied to rows; singular value decomposition with imputation was used to calculate principal components. X and Y axes show principal component 1, and principal component prediction ellipses were such that a new observation from the same group will fall inside the ellipse with probability 0.95. Data distribution normality was tested by Shapiro-Wilk test. Comparison of prepregnancy cytokine levels to the individual trimesters was tested with Wilcoxon matched pairs test. Comparison of cytokine patterns over time between trimesters was performed by analysis of variance (ANOVA) (Friedman test), followed by Dunn's multiple comparison post hoc analysis. Comparisons per time point between patients with IBD and controls or UC versus CD were analysed by Mann-Whitney test. Heat map visualisation was performed using CIMminer (40).

Cytokine microbial network and dynamic analysis

We developed a dynamic model by computing for each two following time points the changes in cytokine levels and in bacteria log frequencies and the tested three correlations:

- Correlation between the (log) level of bacteria at point 1 versus the change in cytokine between point 1 and point 2.
- Correlation between the cytokine level at point 1 versus the change in (log) level of bacteria between point 1 and point 2.
- Correlation between the (log) level of bacteria in point 1 versus the change in (log) level of bacteria between point 1 and point 2.

We then performed the same analysis but scrambled the bacteria. We computed the minimal p values of Pearson coefficients in the scrambled sets to be around 0.01 and thus used 0.01 as the minimal value in all comparisons. A Benjamini-Hochberg correction yielded similar results but may not be appropriate here since the data are not normally distributed.

RESULTS

Characteristics of patients and controls

We first determined basic characteristics of pregnant patients with IBD and normal pregnant controls. For microbiome analysis, 46 patients and 179 controls were included (Table 1). Patients with IBD and controls were of similar age at time of conception and had similar mode of delivery. IBD women used more assisted reproductive technology ($p<0.0001$) and were more often nulliparous ($p<0.0001$). Three patients with IBD used antibiotics during the third trimester for urinary tract or skin infection, with one of these three also using antibiotics during the first trimester. Stillbirth occurred in two patients in trimester 3 (T3). For cytokine levels, a subgroup of 33 patients with IBD and 40 controls was analysed (Table 2). In this subgroup, IBD women were younger at time of conception ($p>0.0001$), had a higher body mass index (BMI) ($p=0.002$) and were more often nulliparous ($p<0.0001$) compared with controls. Mode of delivery and birth outcomes were similar between patients with IBD and controls. Specific patient characteristics are summarised in supplementary Table 1 and 2. Of 19 patients on biologicals prior to pregnancy, 68% stopped this medication after the second trimester (T2). The number of flares (as assessed by clinician, or based on clinical findings, faecal calprotectin and/or endoscopy) during pregnancy did not vary over the course of pregnancy, nor did HBI for CD or SCCAI for UC/IBD unclassified. We concluded that our study group would allow meaningful comparisons between patients with IBD and healthy controls for immunological state and microbiome profiles.

Proinflammatory cytokine levels decrease on conception in patients with IBD and are stable and patient-specific during pregnancy

While altered serum cytokine patterns have been described for non-pregnant patients with IBD, it is as yet unknown whether these patterns are modulated by pregnancy in patients with IBD. We therefore first compared IL-4, IL-5, IL-6, IL-8, IL-9, IL-10, IL-12, IL-15, IL-17, IL-21, TNFα and IFNγ levels in serum obtained preconception and during the three trimesters of pregnancy in a group of 16 patients with IBD for whom paired samples were available (12 patients with CD and patients with 4 UC). PCA analysis of these samples demonstrated that overall cytokine profiles were very similar between patients with CD and UC, with samples taken during inflammation not clustering separately (Figure 1A). We subsequently investigated levels of individual cytokines during pregnancy as compared with prepregnancy. Figure 1B shows that serum levels of IL-6 ($p=0.005$, $p=0.027$, $p=0.012$ for T1–3, respectively), IL-8 ($p=0.042$ for T1, $p=0.012$ for T3), IL-12 ($p=0.034$ for T2), IL-17 ($p=0.0078$ for T2) and TNFα ($p=0.039$ for T2) decreased significantly on conception. In contrast, IL-10 increased from prepregnancy to T1, although not significantly.

Next, we further explored modulation of cytokine profiles in patients with IBD during the three trimesters of pregnancy in a larger group of patients (25 CD, 8 UC, for all measure-

Table 1. Subject characteristics (faecal samples)

		Pregnant IBD patients (n=46)	Pregnant controls (n=179)	p-value
Mean age at conception in years (SD)		29.7 (3.1)	31 (4.1)	0.055
Antibiotic use during pregnancy (%)		3 (6.5)	0	<0.0001
Nulliparous (%)		37 (80.4)	86 (38.4)	<0.0001
Use of assisted reproductive technology (%)		7 (15.2)	6 (2.7)	<0.0001
Delivery (%)	Vaginal delivery Cesarean section	37 (84.1) 7 (15.9)	180 (81.4) 41 (18.6)	0.259
Birth outcome (%)	Live birth Still birth Termination	44 (95.7) 2 (4.3) 0 (0)	221(98.7) 0 (0) 3 (1.3)	0.168 <0.0001 <0.0001

SD: standard deviation

Table 2. Subject characteristics (serum samples)

		Pregnant IBD patients (n=33)	Pregnant healthy controls (n=40)	p-value
Mean age at conception in years (SD)		29.1 (3.5)	32.8 (3.9)	<0.0001
Median pre-pregnancy BMI (IQR)		24.7 (22.7-27.2)	21.6 (19.5-23.7)	0.002
Nulliparous (%)		26 (78.8)	15 (37.5)	<0.0001
Use of assisted reproductive technology (%)		4 (12.1)	2 (5)	0.27
Delivery (%)	Vaginal delivery Cesarean section	25 (75.8) 8 (24.2)	32 (80) 8 (20)	0.663
Birth outcome (%)	Live birth Termination	33 (100) 0 (0)	39 (97.5) 1 (2.5)	0.360
Mean gestational age in weeks (SD)		36.3 (8.4)	39.1 (1.2)	0.104
Birth weight in grams (SD)		3158 (567)	3230 (397)	0.748

IQR: interquartile range; BMI: Body mass index; SD: standard deviation

ments, see online supplementary Figure 1). Only IL-12 and IL-21 for patients with UC, and IFNγ for patients with CD showed modest modulation during the different trimesters of pregnancy (Figure 1C, p=0.0375, p=0.0469 and p=0.0302, respectively). C reactive protein (CRP) levels increased during pregnancy (online supplementary supplementary Figure 1), confirming earlier reports suggesting that CRP cannot be reliably used as a disease activity marker during late pregnancy (40). Direct comparisons between patients with CD and UC indicated lower IL-9 and IFNγ levels in patients with CD as compared with patients with UC in the second (p=0.0272 and p=0.0201) and third trimester (p=0.0361 and p=0.0388).

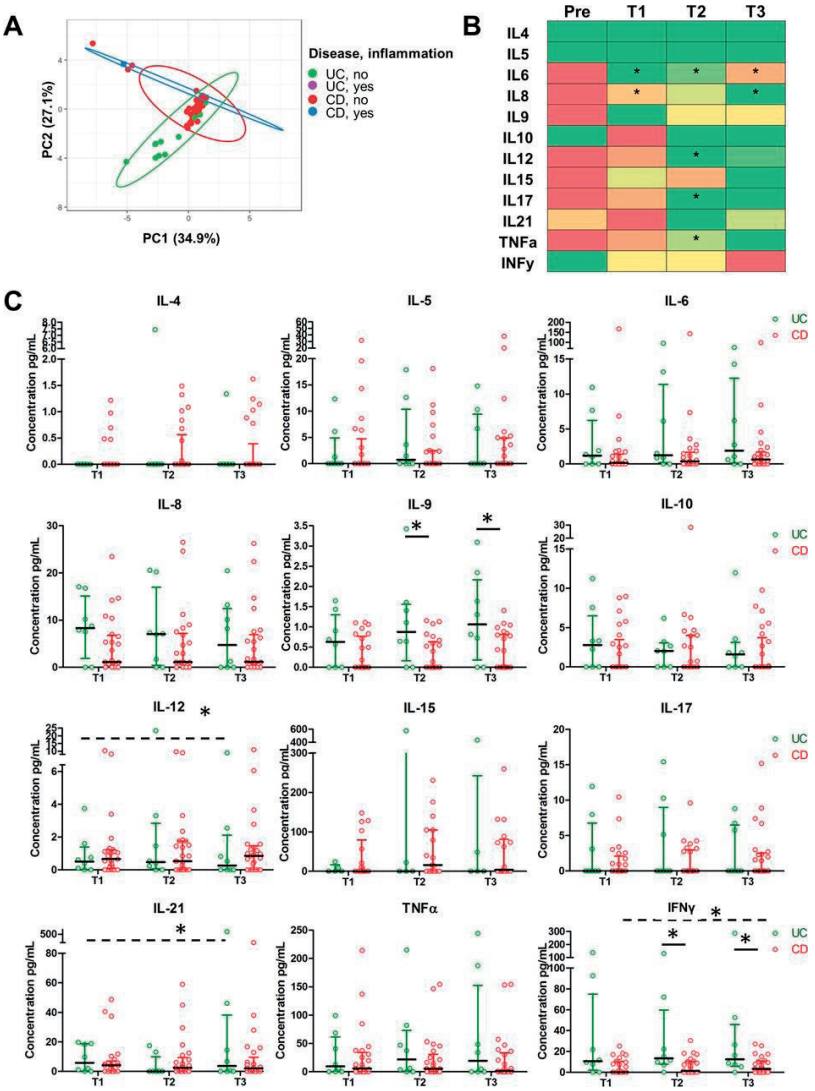

Figure 1. Proinflammatory cytokine serum levels decrease on conception and are stable over time during pregnancy in patients with UC and CD. (A) Principal coordinate analysis shows no overall cytokine changes during pregnancy between patients with UC patients and CD. (B) Median levels of individual cytokines are represented by a colour gradient with green indicating the lowest value for during pregnancy and red indicating the highest value. Significant decreases in several proinflammatory cytokine levels are observed in pregnancy as compared with preconception (indicated by asterisk). With the exception of TNFα, similar significant decreases were seen when patients with CD were analysed separately (not shown). (C) Comparisons of individual cytokines between patients with CD and UC for the three trimesters. Median and IQR are shown. Significant differences (Friedman test, indicated by dashed lines) for different trimesters were observed for patients with UC for IL-12 and IL-21. Significant differences between patients with UC and CD in a given trimester were seen for IL-9 and IFNγ (Mann-Whitney test, indicated by solid lines). CD, Crohn's disease; IL, interleukin.

Medication use changes during pregnancy. Most noticeably, anti-TNFα use is decreased in T3, with more patients using no medication at all at this time point (supplementary Figure 2A). Usage of 5-aminosalicylic acid (ASA) and thiopurines did not fluctuate over time. PCA indicates that different treatments are not associated with an overall altered cytokine profile (supplementary Figure 2B). We next investigated whether treatment affects individual serum cytokine levels. ANOVA showed no overall differences between the different treatment regimes in terms of cytokine expression (supplementary Figure 2C). However when compared only with unmedicated patients, patients on 5-ASA showed lower IL-10 (p=0.0267) and IL-17 (p=0.0428) levels. Patients on anti-TNFα single treatment showed significantly lower IL-8 levels as compared with untreated patients (p=0.001). None of the patients stopping anti-TNFα in our cohort experienced a flare. Overall, cluster analysis showed that grouping of samples was more dependent on the individual patients rather than the disease manifestation, the presence or absence of intestinal inflammation and the pregnancy trimester from which the sample was obtained or the medication used (supplementary Figure 3). Thus, serum proinflammatory cytokine levels (and hence overall immunological state) in patients with IBD decrease on conception and are relatively stable during pregnancy.

IL-10 and IL-6 cytokine levels increase over time in healthy pregnancies but not IBD

Next, we investigated whether serum cytokine levels behave differently in patients wtih IBD and healthy controls during pregnancy. PCA indicated that overall cytokine profiles did not shift over time in healthy pregnancy (Figure 2A) and that overall cytokine profiles in this panel were similar when comparing patients with IBD and controls (Figure 2B). However, analysis of individual cytokines showed that serum levels of IL-6, IL-10 and TNFα changed significantly over the three trimesters in healthy pregnant women (Figure 2C, p=0.0124, p=0.0458 and p=0.0030, respectively). Most noticeably, both IL-6 and IL-10 levels showed a significant upregulation towards the third trimester in the healthy controls. However, this upregulation was not seen in pregnant patients with IBD, with IL-10 levels lagging significantly behind healthy controls in the second and third trimester (p=0.0385 and p=0.0016, respectively). In addition, significantly reduced IL-5 levels were observed in patients with IBD during the entire pregnancy (p=0.0194 for T1, p=0.0368 for T2, p=0.0228 for T3). In contrast, IL-8 and IFNγ levels were increased as compared with controls (IL-8: p<0.0001, p=0.0002 and p=0.0003 for T1 and T3, IFNγ: p=0.0443 for T2, p=0.0130 for T3). Patients with IBD only showed overall differences in IL-9 levels throughout pregnancy (p=0.0326). Thus, pregnancy in healthy women is associated with specific changes in peripheral blood cytokines that seem to be largely absent in expecting patients with IBD.

The microbiome of pregnant women with IBD differs between patients with CD and UC and is affected by disease location in CD

We then analysed the microbiota of all patients with IBD throughout pregnancy. Beta diversity (between sample) analysis did not reveal any significant differences relating to the dif-

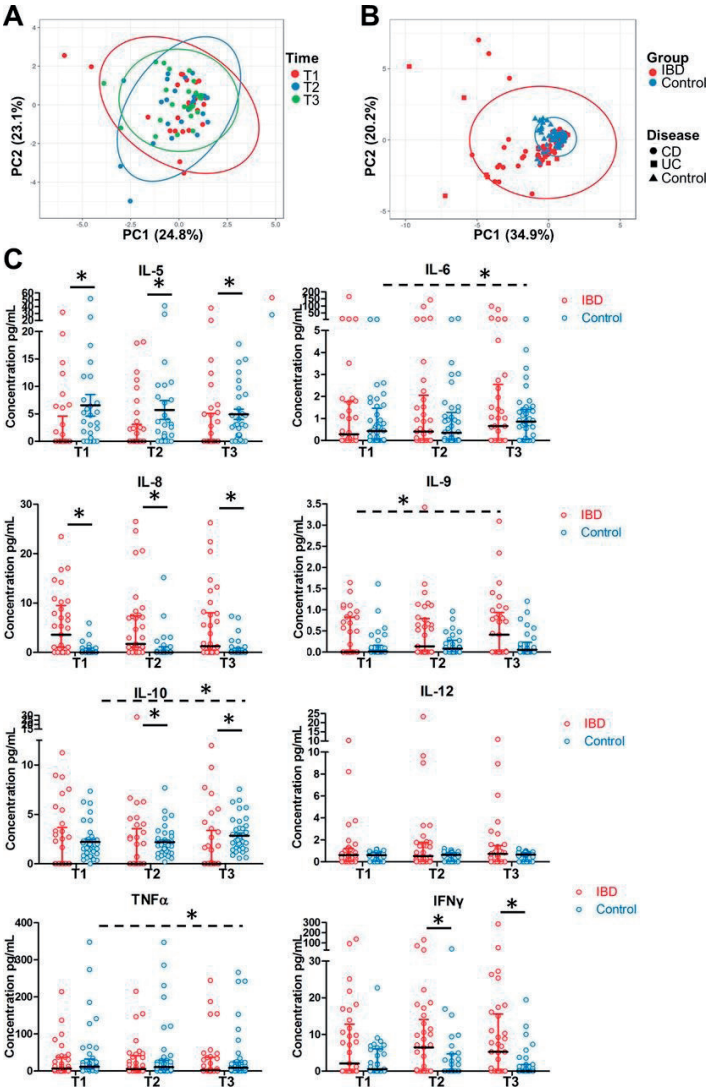

Figure 2. IL-6 and IL-10 serum levels rise during pregnancy in healthy controls but not patients with IBD. (A) Principal coordinate analysis (PCA) showing lack of overall cytokine changes during healthy pregnancy. (B) PCA showing that overall cytokine patterns do not cluster separately between patients with IBD and controls. Samples from first, second and third trimester were included in the analysis. (C) Comparisons of individual cytokines between patients with IBD and healthy controls for all three trimesters. Median and IQR are shown. Significant differences (Friedman test, indicated by dashed lines) for different trimesters were observed in healthy controls for IL-6, IL-10 and TNF-α and for IL-9 in patients with IBD. Significant differences between patients with IBD and healthy controls in a given trimester were seen for IL-5, IL-8, IL-10 and IFNγ (Mann-Whitney test, indicated by solid lines). IL, interleukin.

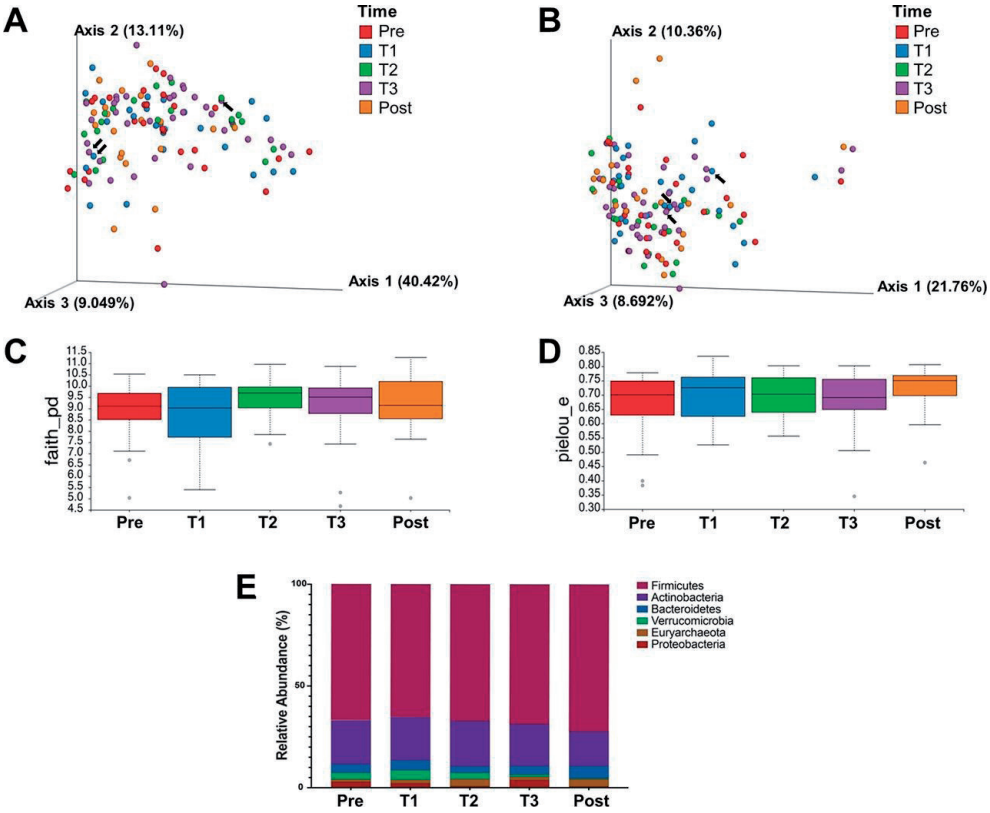

Figure 3. Microbial diversity parameters of patients with IBD do not change throughout pregnancy. Faecal samples from patients with IBD were collected at five time points: prepregnancy, first, second, third trimester and postpartum (27, 27, 21, 36 and 19 samples, respectively). (A and B) β-diversity using principal coordinate analysis of unweighted (A) and weighted (B) UniFrac distances. The black arrows point to samples of patients who used antibiotics. (C and D) α-Diversity using Faith's phylogenetic diversity (C) and Pielou's evenness plot (D) measurements. (E) Taxonomy plot at the phylum level.

ferent time points or use of antibiotics (Figure 3A,B). The richness (Figure 3C) and evenness (Figure 3D) as measured by Faith's PD and Pielou respectively also did not differ significantly over time. When looking at the relative abundance (Figure 3E), Firmicutes tended to increase as pregnancy progressed and Actinobacteria and Verrucomicrobia decreased, although not significantly.

Figure 4 summarises the differences between women with CD and women with UC. When classifying patients with CD and UC, the area under curve was 0.75 for the test results, showing that the microbiome reflects disease type (Figure 4A). Spectral clustering of the bacteria demonstrated differences in bacterial communities between patients with CD and UC (Figure 4B) and a more specific analysis showed significant differences in several bacteria

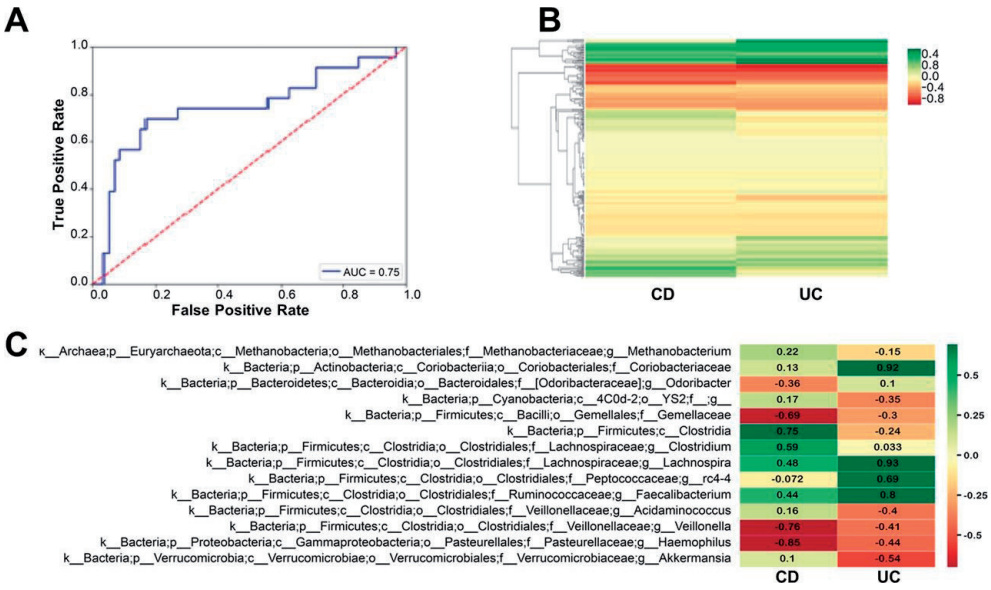

Figure 4. Overall microbiome composition of patients with CD differs from patients with UC. (A) ROC curve of classification of UC (n=41) versus CD (n=89). (B) Spectral clustering of bacteria based on the bacterial differences between the UC and CD. (C) Bacteria with differences between UC and CD with significance with p<0.05. The marked values are the average (10log) value for each bacterium in each group. CD, Crohn's disease; ROC, receiver operating characteristic.

that behaved differently between the two diseases (Figure 4C). For example, *Methanobacterium*, *Acidaminococcus* and *Akkermansia*, an unclassified member of the YS2 order and an unclassified member of the Clostridia class, were all positively correlated with the CD microbiome and negatively correlated with the UC microbiome. *Odoribacter* and an unclassified member the family Peptococcaceae showed the opposite behaviour (Figure 4C).

Having established that UC and CD patients' microbial signatures are distinctive, we next asked whether disease location in patients with CD would also affect microbial composition. Unweighted UniFrac demonstrated significant differences in faecal microbiome between patients suffering from colonic versus non-colonic disease (p=0.011) (Figure 5A). In addition, we found significant feature differences. In patients with exclusive colonic disease, features such as *Bifidobacterium*, *Turicibacter*, *Clostridium*, *Oscillospira* and *Dialister* were highly abundant. In the patients with ileal or ileocolonic disease, the features *Methanobrevibacter* and *Ruminococcus* were more abundant (Figure 5B). Alpha diversity was higher in samples from patients with colonic disease by using Pielou's Evenness plot (p=0.02) and Shannon's diversity index (0.043) (Figure 5C,D). Colonic disease in patients with CD is not similar to UC disease: significant differences were seen when comparing colonic and non-colonic CD patient samples to UC samples (unweighted UniFrac beta diversity differences, p=0.035 and p=0.028, respectively, data not shown).

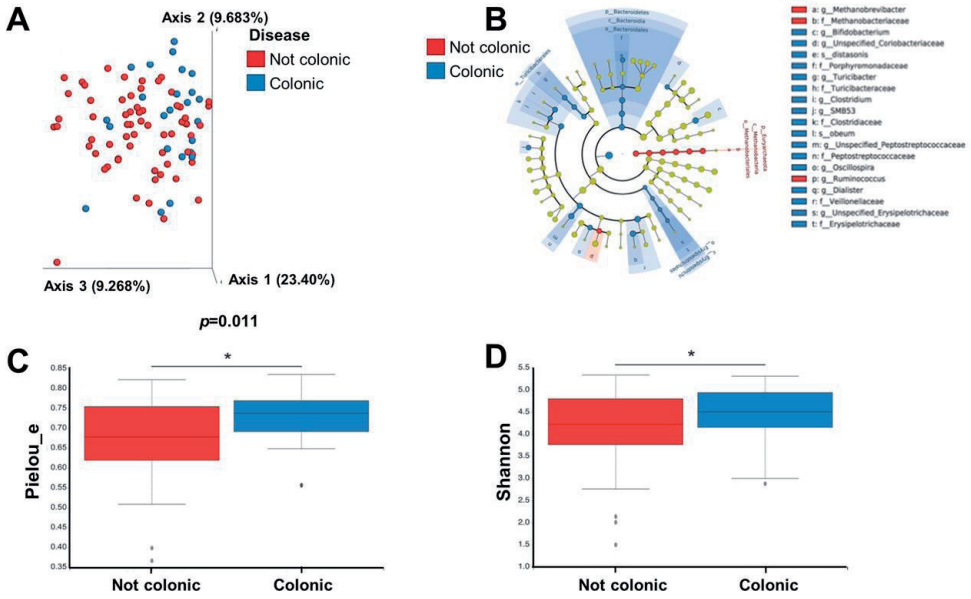

Figure 5. The microbiome of patients with CD suffering from colon disease is different than that of patients with non-colonic disease. Patients with CD were divided according to disease location: colonic (n=24) and not colonic (n=65). (A) β-diversity using principal coordinate analysis of unweighted UniFrac distances (p=0.011) (B) Significantly abundant taxa in each of the groups by LEfSe analysis. (C and D) α-diversity using (C) Pielou's evenness plot (p=0.02) and (D) Shannon's diversity index (p=0.043). LEfSe, linear discriminant analysis effect size.

When comparing the differences between patients with UC and CD at a given time point, significant differences in beta diversity were observed in the prepregnancy samples (p=0.041) in unweighted and weighted UniFrac (Figure 6A,B), but not at T1, T2 or T3 or postpregnancy (data not shown). LEfSe analysis, which is based on LDA scores highlighted the significant features at each time point (Figure 6C–G). Before initiation of pregnancy, women with UC had higher abundance of *Bifidobacterium adolescentis* compared with women with CD, who had higher abundances of *Ruminococcus gnavus* and *Escherichia coli* (Figure 6C). In early pregnancy, women with UC had an over-representation of *Bacteroides caccae* and the genus *Odoribacter*, whereas women with CD had increased levels of *Blautia obeum* (Figure 6D). The higher levels of *E. coli* seen in prepregnant women with CD appeared again in T2. Women with UC had higher levels of the genera *Actinomyces*, *Anaerostipes* and *Veillonella* (Figure 6E). *Veillonella* remained significantly higher in these women in T3 as well, and this was accompanied with higher abundance *Blautia* and unclassified members of the Clostridiaceae and Lachnospiraceae compared with the CD microbiome in T3, which had higher levels of *F. prausnitzii* and *Ruminococcus bromii* (Figure 6F). *R. bromii* remained over-represented postpregnancy too (in CD patients), as opposed to *Bacteroides ovatus*, Streptococcus and an unclassified member of Lachnospiraceae (higher in T3 as well) that increased postpartum in women with UC (Figure 6G). A centroid-based clustergram

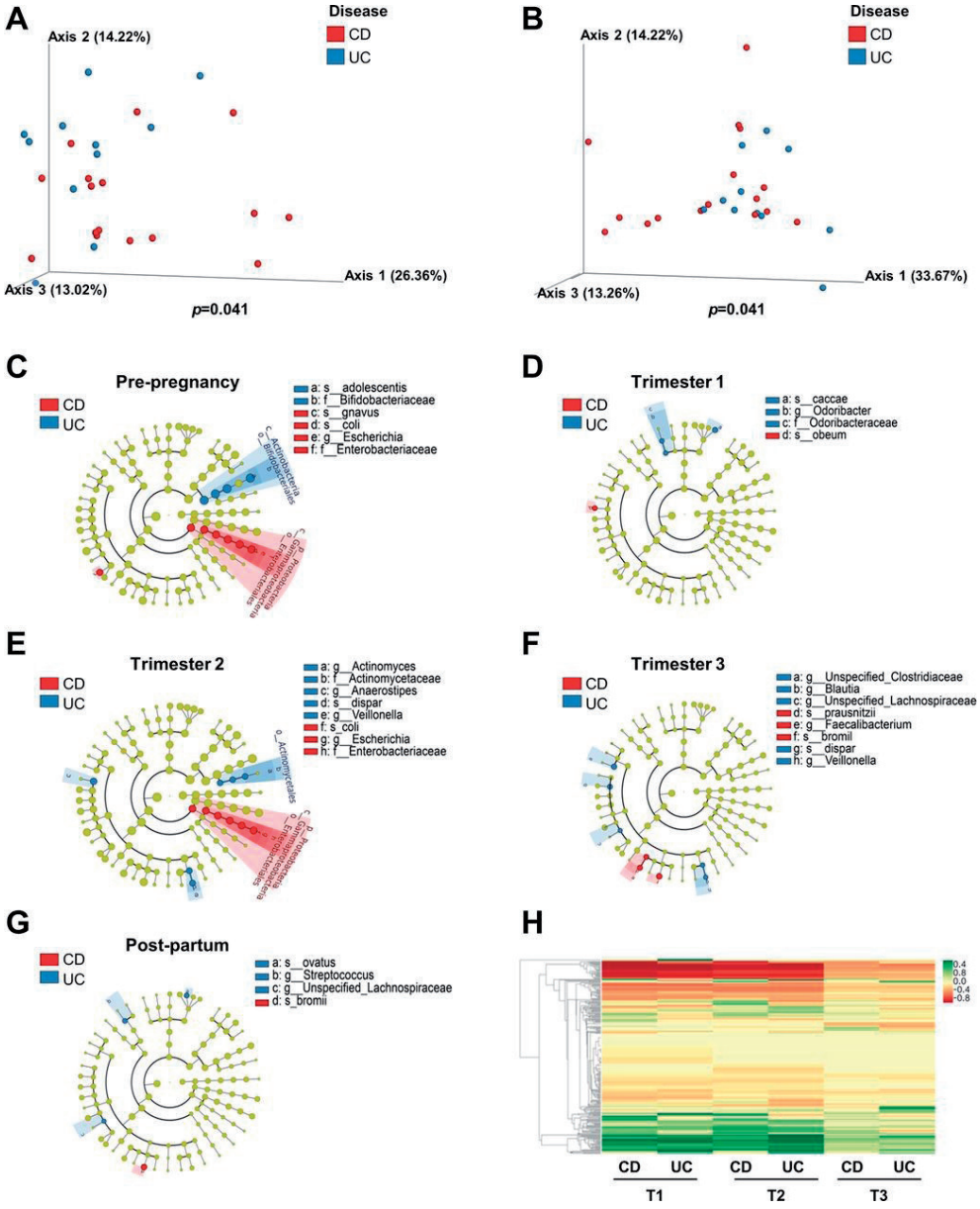

Figure 6. The microbiomes of patients with CD and UC are dominated by different species at each time point. (A and B) β-diversity using principal coordinate analysis of unweighted (A) and weighted (B) UniFrac distances (p=0.041) of prepregnancy samples (CD n=16, UC n=11). (C–G) Cladogram of significant differentially abundant microbial taxa obtained using LEfSe of prepregnancy (C), first (CD n=19, UC n=8) (D), second (CD n=16, UC n=5) (E), third (CD n=25, UC n=11) (F) trimester and postpartum (CD n=13, UC n=6) (G) gut microbiomes. (H) Spectral clustering of bacteria based on the difference between the UC and CD and over all trimesters. CD, Crohn's disease; LEfSe, linear discriminant analysis effect size.

(Figure 6H) allows for visualisation of the differences between the two IBD states and of those observed at the different time points related to pregnancy.

The effect of inflammation and medication on microbial signatures during pregnancy in IBD

Differences in the microbiome of patients that experienced a flare during pregnancy compared with those who did not were also analysed for patients with CD and UC separately. Pregnant women with CD had significantly higher bacterial evenness (p=0.025) and richness (p=0.03, Figure 7A) when experiencing a flare. LEfSe analysis demonstrated several features that were over-represented in women with a flare. These included *Collinsella aerofaciens*, *Bacteroides ovatus*, *Dorea formicigenerans*, *Bilophila* and the phylum Bacteroidetes in general (Figure 7B). For patients with UC, no significant differences were found in richness and evenness measurements (supplementary Figure 4A,B), although LEfSe analysis indicated that women suffering from a flare had higher relative abundance of *Odoribacter*, *Bilophila* and *Parabacteroides distasonis*, whereas the microbiome of women who did not suffer from a flare was enriched with *Coprococcus*, *Lachnospira* and *F. prausnitzii*.

Next, we analysed the effect different medications had on the microbiome. LEfSe analysis revealed multiple features that were different between the different treatments, which are summarised in Figure 7C. Interestingly, *Coprococcus catus* and *Ruminococcus torques* were over-represented in women with CD who did not receive any medication. No significant differences were observed for different treatments in patients with UC. We also examined the effect of BMI and did not observe any influence on β-diversity or α-diversity. However, we did find two taxa that differed (*Dorea formicigenerans* was more overrepresented in BMI <25 and *R. bromii* was overrepresented in BMI >25, data not shown).

Parity influences the microbiome in pregnant patients with IBD

The microbiome of multiparous women with IBD compared with nulliparous women exhibited significant differences in beta diversity in unweighted and weighted UniFrac (p=0.027 and p=0.045, respectively) (Figure 8A,B). In addition, nulliparous patients had higher bacterial richness (Figure 8C). Several features were highly abundant in nulliparous samples as found by LEfSe analysis such as *Anaerostipes* and *Oscillospira*. However, *Bacteroides* and *Bilophila* were more abundant in multiparous patients (Figure 8D). No differences were seen on cytokine patterns between IBD women who had or who had not born children before (not shown).

The microbiome from pregnant patients with IBD is less rich and more similar than microbiome of pregnant healthy controls

Since the two cohorts originated from different countries and were extracted using different kits, we only compared diversity indices as we have shown previously that this to be

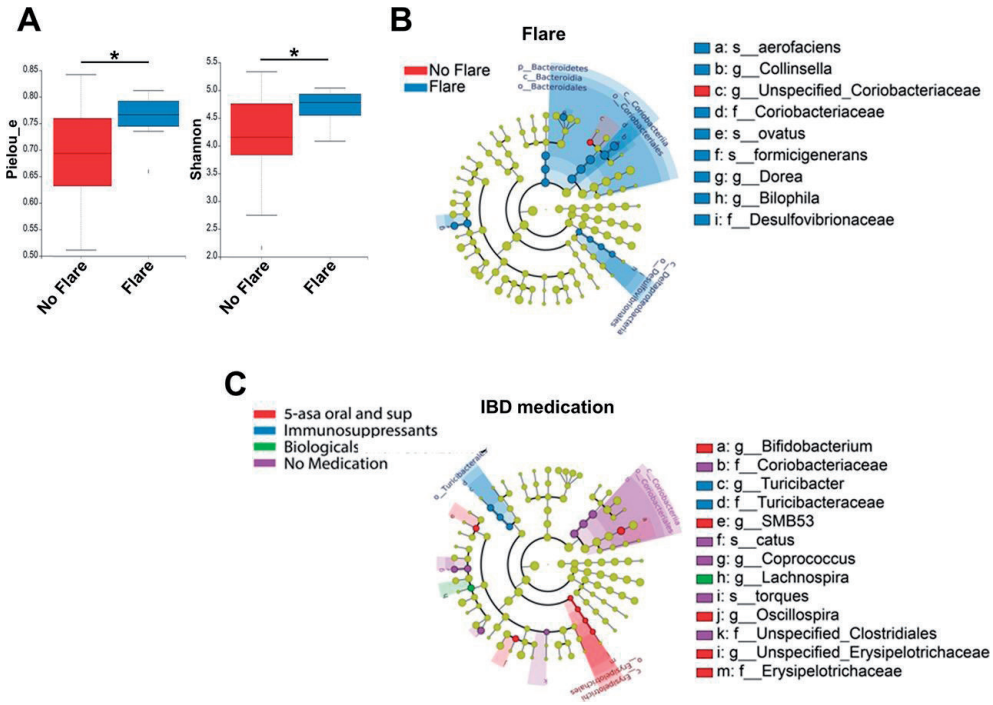

Figure 7. Microbiota of patients with CD differs when suffering from a flare. Patients were divided according to those who suffered a flare (eight samples) during gestation compared with those with stable disease (51 samples) and by IBD medication: 5-ASA oral and sup, immunosuppressants, biologicals and no medication (3, 14, 12 and 23 samples, respectively). (A) α-Diversity using Pielou's evenness plot (p=0.025) and Shannon's diversity index measurements (p=0.03) comparing flare and no flare samples. (B and C) Cladogram of significantly differentially abundant microbial taxa obtained using LEfSe divided by flare occurrence (B) or IBD medication (C). CD, Crohn's disease.

a valid strategy. (19) The microbiota of patients with IBD was more similar to one another (beta diversity) both by unweighted (Figure 9A; p=0.001) and weighted UniFrac (Figure 9B; p=0.001) than that of healthy controls. Patients with IBD also had lower bacterial richness as measured by Faith's PD (Figure 9C; p<0.001) and evenness as measured by Pielou's evenness (Figure 9D; p=0.008). We also compared the alpha diversity differences per trimester. Patients with IBD showed a significantly reduced bacterial richness in T1 compared with the controls (Figure 9E; p=0.001), which may even be underestimated, as patients with IBD were also more often nulliparous and nulliparity was associated with higher alpha diversity in these patients. This effect disappeared later in pregnancy. Exclusion of comorbidities did not alter these results.

Cytokine microbial network and dynamic analysis

As summarised in Figure 10, *Sutterella* was the hub of a bacterial network. This means that the abundance of multiple features was positively correlated to an increase in the abun-

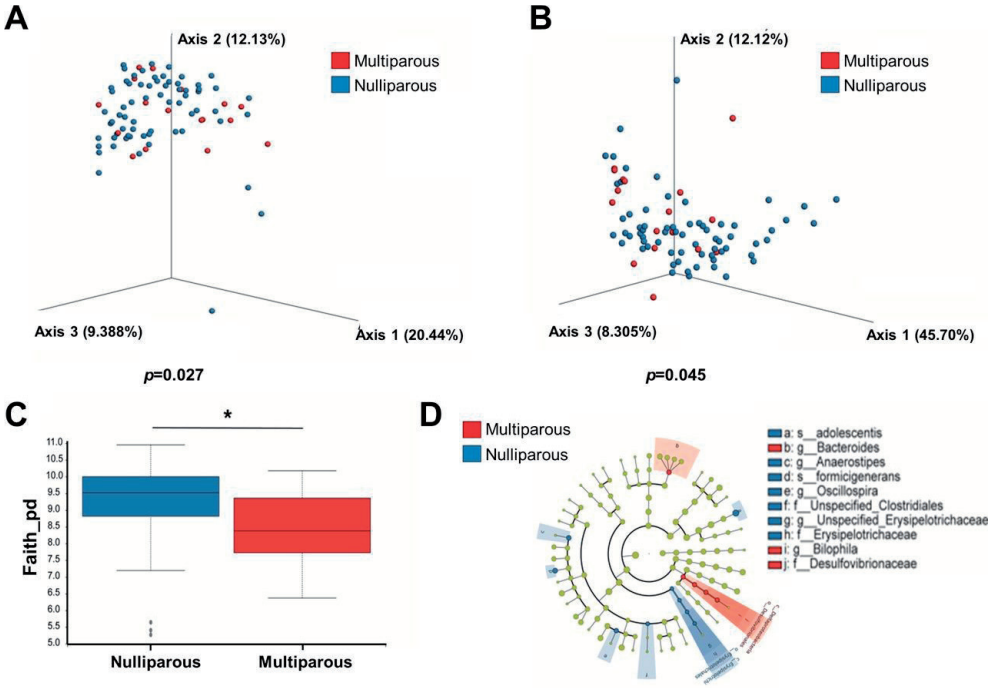

Figure 8. The microbiome of multiparous pregnancies is different from nulliparous pregnancies. Faecal samples collected during gestation were divided according to patients' previous pregnancies into nulliparous (n=67) versus multiparous (n=17). (A and B) β-diversity using principal coordinate analysis of unweighted (A) and weighted (B) UniFrac distances (p=0.027 and p=0.045, respectively). (C) α-Diversity using Faith's phylogenetic diversity (p=0.017). (D) Differently abundant taxa in each of the groups by LEfSe analysis.

dance of *Sutterella* at a later timepoint. We also observed negative correlations between bacteria, for example, the abundance of *Faecalibacterium* was correlated to a decrease in abundance of *Roseburia* at a second timepoint. When modelling the interactions between levels of cytokines and bacterial abundance, we again observed positive and negative correlations. For example, the levels of IL-9 and IL-17 were both correlated with a decrease in the abundance of an unclassified genus of the Rikenellaceae, whereas IL-5 was positively correlated with an increase in the abundance of *Akkermansia* and *Ruminococcus*. Overall, these analyses provide a wealth of data detailing the correlations between microbiome, disease type, cytokine profiles and pregnancy.

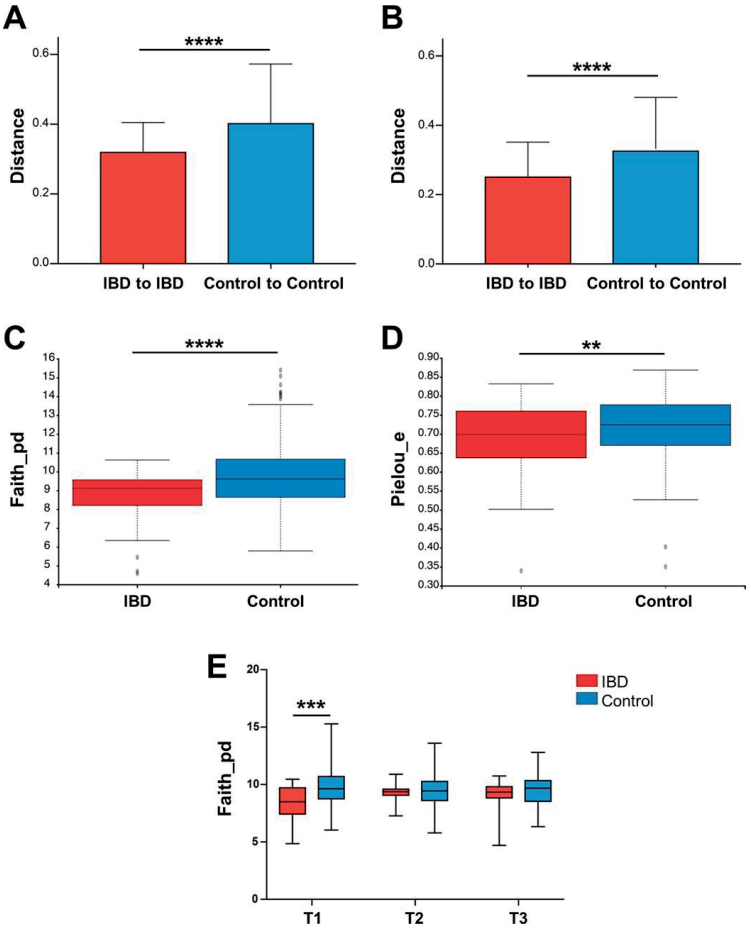

Figure 9. Patients with IBD have more uniform microbiomes than healthy controls. A comparison of the gut microbiomes of IBD (130 samples) and control (236 samples) pregnant women. (A and B) Beta-diversity of unweighted (A) and weighted (B) (p=0.001) UniFrac distances. (C and D) α-Diversity using (C) Faith's phylogenetic diversity (p<0.0001), and (D) Pielou's evenness plot (p=0.008) measurements. (E) Faith's phylogenetic diversity comparing IBD and control samples by pregnancy trimesters (p=0.0004).

DISCUSSION

Normal pregnancy is associated with hormonal, microbial and immunological changes, which prepare the maternal body for successful childbirth. Interestingly, some autoimmune diseases, including rheumatoid arthritis and multiple sclerosis, show improvement during pregnancy, while risk of flares increases postpartum (14-16). This suggests that pregnancy-induced physiological changes affect immune processes at peripheral sites, and it has been suggested that increased levels of Tregs and a shift towards Th2 cytokine patterns

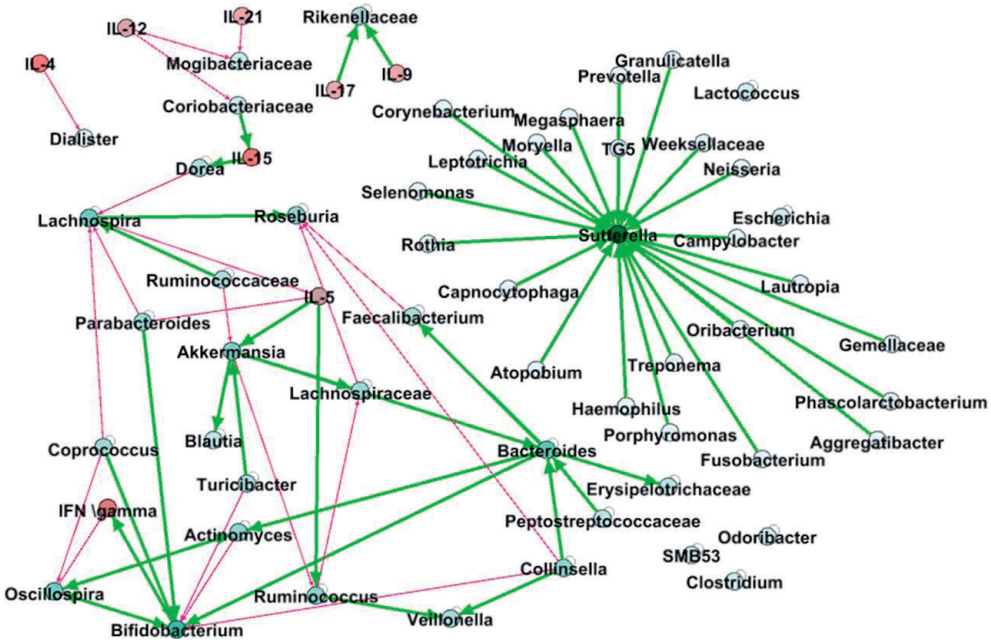

Figure 10. Correlation between current value of the source node and the change in the value of a target node. Thick green arrows represent positive correlations, while thin red arrows represent negative correlations. A thin grey round arrow represents a correlation between the current value of a feature and future change. Cytokines are marked in red nodes.

contribute to amelioration of Th1-driven diseases (41,42) and conversely also play a role in exacerbation of Th2-driven inflammatory diseases such as systemic lupus erythematosus during pregnancy (43,44). CD is generally thought to constitute a Th1/Th17 disease, while UC is more Th2/Th17 driven, which may explain differences in disease patterns during pregnancy observed between these types of IBD (10). However, while surprisingly little consensus has been reached for other cytokines, one of the most consistent findings in healthy women are rising peripheral blood levels of IL-6 (generally considered a Th1 cytokine) from early to late pregnancy (18,45-47). Our own results confirm that changes in cytokine patterns from T1 to T3 are present during healthy pregnancy. However, it was not possible to ascribe our observed changes during pregnancy to either Th2 cytokines (eg, IL-4, IL-5 and IL-13) or cytokines commonly associated with Th1 responses (IFNγ and TNFα). While we observed increased levels of IL-6 and reduced expression of TNFα during late gestation in healthy females, both of these have been ascribed to both Th1 and Th2 cells as well as a range of other cell types (48). Thus, based on our data, it is unlikely that an outspoken Th2 shift during pregnancy ameliorates (auto-)immune diseases. However, in the current study, we do show that several proinflammatory cytokines (IL-6, IL-8, IL-12, IL-17 and TNFα), known to play a role in IBD pathophysiology (49,50), decrease significantly on conception, suggesting that pregnancy reduces immunological parameters of inflammation in patients

with IBD. During pregnancy itself, serum cytokine levels in patients with IBD subsequently remained relatively stable, with reduced levels of IL-5 and IL-10 levels and increased IL-8 and IFNγ levels compared with control, throughout the three trimesters. Overall, it seems that the immunological state of patients with IBD improves on pregnancy.

Differences between CD and UC microbiomes have been reported previously (51), and accordingly in this study, we could differentiate between CD and UC solely based on the microbiomes with an area under the curve (AUC) of 0.75. Here, we studied the effect of pregnancy on the UC and CD microbiomes as we know that pregnancy influences the microbiota. The microbiomes of patients with CD and UC remained different from each other throughout pregnancy. However, while before pregnancy, beta diversity differed between patients with UC and CD, the onset of pregnancy caused a shift in beta diversity, which caused the microbiomes to behave similarly diversity wise. In general, it seems that as pregnancy progressed changes in bacterial features became more subtle (Figure 6H), suggesting a dampening effect of pregnancy on microbial differences. Indeed very few patients experienced relapse of disease during pregnancy. Those patients with CD experiencing a flare had a significantly less diverse and evenly distributed microbiome than patients with CD who did not experience a flare. At the genus level, the only genus increased in both patients with CD and UC who suffered from a flare at any point during pregnancy was *Bilophila*, which has been shown to increase under inflammatory and pathological conditions such as inflammatory disorders and appendicitis (52) and has been suggested to be involved in the initiation of IBD(53). However, the butyrate producing *F. prausnitzii* was over-represented in patients with UC with no relapse. *F. prausnitzii* is considered to have anti-inflammatory properties which may help dampen the flaring process and has even been considered to have a clinical potential in IBD (54).

Two of the main microbiome characteristics observed in both disease and pregnancy are lower alpha diversity and greater beta diversity (19,55). The comparison of IBD with healthy microbiomes revealed that the IBD microbiomes were less diverse and even than the healthy controls. This trend of lower diversity in patients with IBD has been previously reported (51,56) and was expected. To our surprise, we observed that the IBD microbiomes were more similar to one another (lower beta diversity), suggesting that the same species are disappearing during disease from the majority of patients. We could not identify which bacteria differed between the two cohorts as the two cohorts are from different countries and the DNA was isolated using different protocols, and we have previously shown that samples extracted via different methods still show the same diversity patterns but might change at feature levels (19). Nevertheless, we have previously demonstrated that during pregnancy in healthy females microbial diversity decreases (19). The fact that alpha diversity differed between patients with IBD and controls during early pregnancy but decreased at later gestational times indicates that pregnancy in IBD is not followed by an additional

loss of diversity on top of the already altered microbial composition in these patients.

In order to produce a dynamic model, we tested the correlation between changes in cytokine or bacteria log levels between two time points and the value of all cytokines and log bacteria expression in the initial time. While these correlations are not a clear evidence of causality since common cause effects can ruin causality, they provide a first order dynamic model. Of great interest was *Sutterella*, which increased in correlation to several other bacteria. *Sutterella* has previously been shown to be increased in patients with IBD compared with healthy controls (57). The negative correlation between *Oscillospira* and IFNγ has been reported previously in mice (58). Oscillospira is a known butyrate producer and has been shown to be decreased in inflammatory states (59). The interaction between *Oscillospira* and *Actinomyces* is also worth mentioning as the first was described to be decreased in IBD (57), whereas the latter has been shown to increase in patients with IBD (60). The association between the abundances of *Faecalibacterium* and *Roseburia* and IBD was also described previously (61), but our model demonstrates that the abundance of *Faecalibacterium* is associated with the decrease in abundance of *Roseburia*.

Thus, *in toto*, these data suggest that immunological parameters improve in patients with IBD on pregnancy, while microbial diversity normalises to that seen in healthy pregnancy.

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Supplementary Table 1. Patient characteristics (fecal samples)

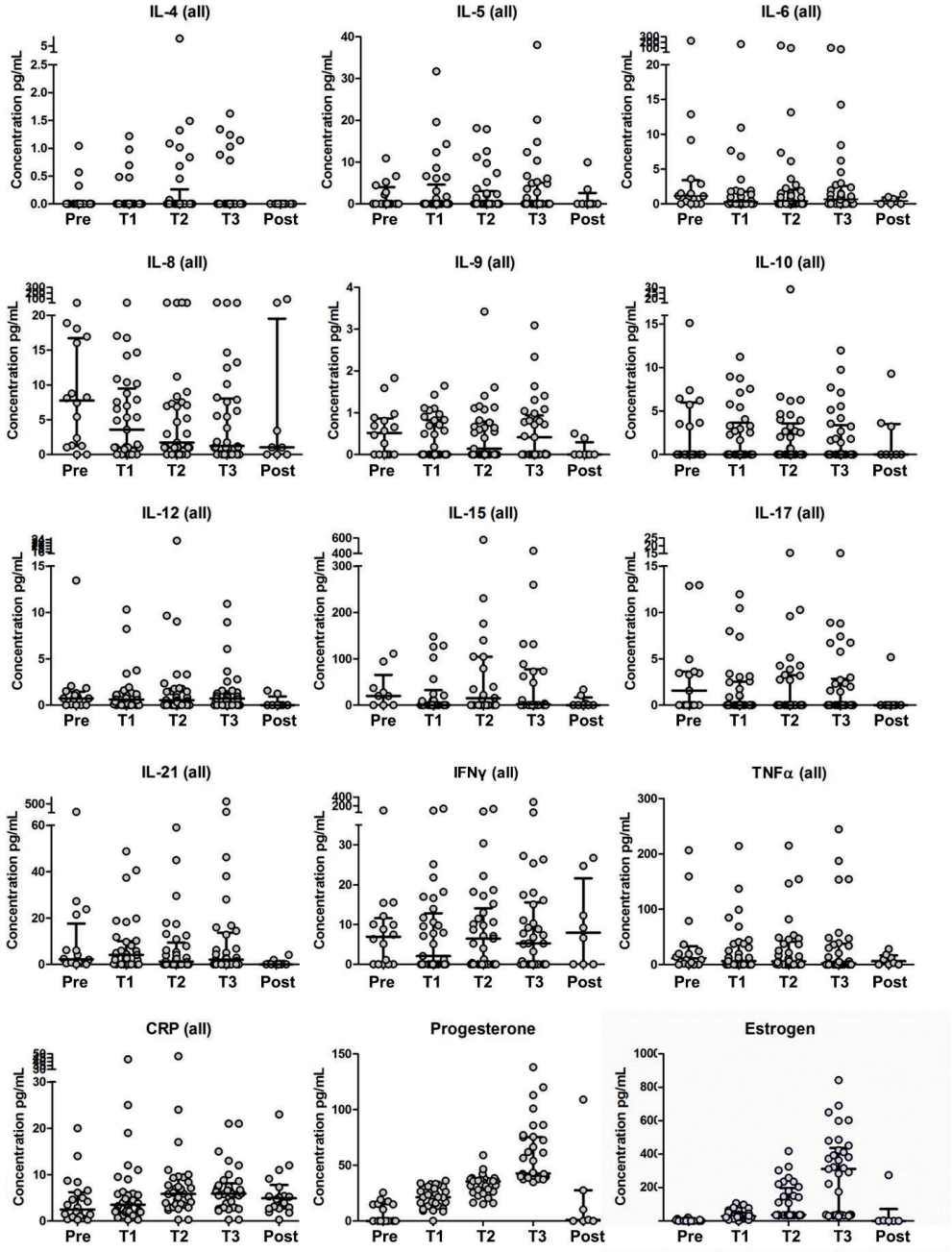
		pre-pregnancy	T1	T2	T3	post-partum	P-value
Mean age at diagnosis in years (SD)		22.6 (3.7)					
Mean weeks pregnant at collection (SD)			10.3 (3)	19.7 (3)	31.9 (3.3)		
IBD diagnosis	UC/IBD-U (%) CD (%)	15 (32.6) 31 (67.4)					
Disease location CD (Montreal) (%)	L1 Ileal L2 Colonic L3 Ileocolonic	5 (16.1) 8 (25.8) 18 (58.1)					
Disease behaviour CD (Montreal) (%)	B1 Non stricturing non penetrating B2 Stricturing B3 Penetrating B2+B3 Strictiring and penetrating	23 (74.2) 2 (6.5) 4 (12.9) 2 (6.5)					
Disease extent UC/IBD-U (Montreal) (%)	E1 Proctitis E2 Left-sided colitis E3 Pancolitis	2 (13.3) 7 (46.7) 6 (40)					
Median HBI for CD (IQR)			1 (0-2.5)	0.5 (0-2)	0 (0-2.5)		.6763 (Kruskal Wallis)
Median SSCAI for UC/IBD-U			1 (0-4)	1 (0.5-4.5)	1 (0.5-1.5)		.8070
Median disease duration in years (IQR)		5 (2-9)					
IBD maintenance medication pre-pregnancy (monotherapy)	None 5-ASA Steroids Thiopurine Anti-TNF/anti-integrin	9 (19.6)7 (15.2) 1 (2.2)8 (17.4) 13 (28.3)	11 (23.9)4 (8.7) 1 (2.2)7 (15.2) 12 (26.1)	11 (23.9)4 (8.7)07 (15.2) 12 (26.1)	20 (43.5)4 (8.7) 1 (2.2)9 (19.6) 4 (8.7)	10 (21.7)6 (13) 07 (15.2)12 (26.1)	
IBD maintenance medication pre-pregnancy (combination)	Anti-TNF + thiopurines Anti-TNF + thiopurine + 5-ASA Anti-TNF + steroids Anti-TNF + steroids + 5-ASA Thiopurine + 5-ASA 5-ASA + steroids	3 (6.5) 0 1 (2.2) 2 (4.3) 2 (4.3) 0	2 (4.3) 2 (4.3) 2 (4.3) 0 3 (6.5) 2 (4.3)	2 (4.3) 2 (4.3) 2 (4.3) 0 3 (6.5) 2 (4.3)	0 1 (2.2) 1 (2.2) 0 4 (8.7) 2 (4.3)	2 (4.3) 2 (4.3) 2 (4.3) 0 3 (6.5) 2 (4.3)	
IBD surgery (%)	Bowel resection Perianal surgery Pouch Appendectomy	6 (13) 2 (4.3) 1 (2.2) 1 (2.2)					
Flare (%)		3 (6.5)	5 (10.9)	6 (13)	3 (6.5)	3 (6.5)	.7198

CD: Crohn's disease; UC: ulcerative colitis; CD: standard deviation; IQR: interquartile range; y: years; IBD: inflammatory bowel disease; HBI: Harvey Bradshaw index; SCCAI: simple clinical colitis activity index; TNF: tumor necrosis factor; ASA: aminosalicylic acid

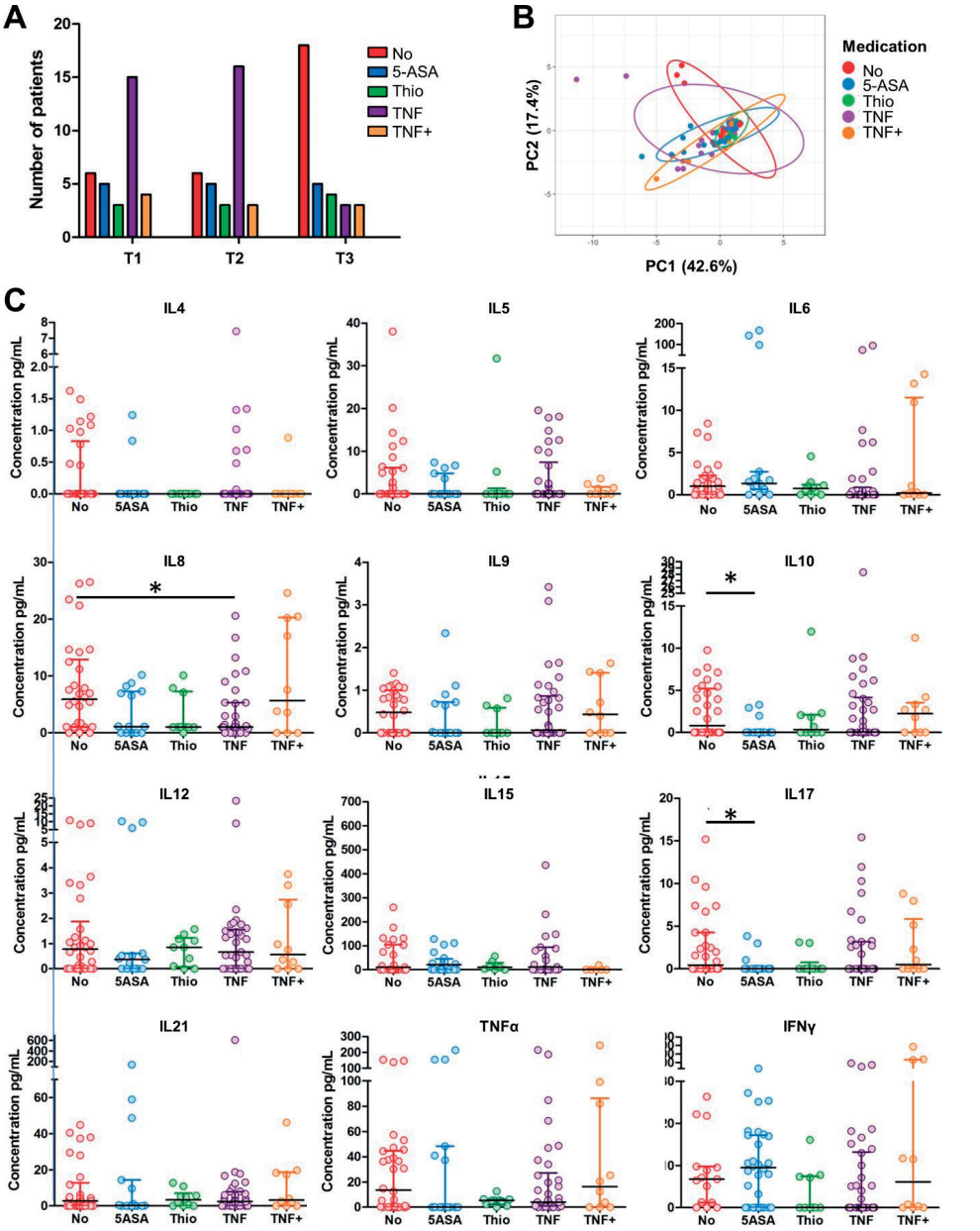
Supplementary Table 2. Patient characteristics (serum samples)

		pre-pregnancy	T1	T2	T3	post-partum	P-value
Mean age at diagnosis in years (SD)		22.8 (4.9)					
Mean weeks pregnant at collection (SD)			8.4 (2.1)	21.1 (2.6)	32 (3)		
IBD diagnosis	UC/IBD-U (%)	8 (24.2)					
	CD (%)	25 (75.8)					
Disease location CD (Montreal) (%)	L1 Ileal	2 (8)					
	L2 Colonic	11 (44)					
	L3 Ileocolonic	12 (48)					
Disease behaviour CD (Montreal) (%)	B1 Non stricturing non penetrating	16 (64)					
	B2 Stricturing	2 (8)					
	B3 Penetrating	3 (12)					
	B2+B3 Strictiring and penetrating	4 (16)					
Disease extent UC/IBD-U (Montreal) (%)	E1 Proctitis	1 (12.5)					
	E2 Left-sided colitis	3 (37.5)					
	E3 Pancolitis	4 (50)					
Median HBI for CD (IQR)			3 (1-5.5)	1 (0-3.5)	1 (0-2.5)		.3331 (Kruskal Wallis)
Median SSCAI for UC/IBD-U (IQR)			4 (0.5-5.5)	4 (0-5)	1 (1-2)		.7479
Mean disease duration in years (SD)		6.5 (4.8)					
IBD maintenance medication pre-pregnancy (monotherapy)	None	3 (9.1)	6 (18.2)	6 (18.2)	18 (54.5)	6 (18.2)	
	5-ASA	7 (21.2)	5 (15.2)	5 (15.2)	5 (15.2)	5 (15.2)	
	Thiopurine	3 (9.1)	2 (6.1)	2 (6.1)	3 (9.1)	2 (6.1)	
	Anti-TNF/anti-integrin	15 (45.5)	15 (45.5)	16 (48.5)	3 (9.1)	16 (48.5)	
IBD maintenance medication pre-pregnancy (combination)	Anti-TNF + thiopurine	1 (3)	1 (3)	1 (3)	0	1 (3)	
	Anti-TNF + steroids	2 (6.1)	1 (3)	0	1 (3)	0	
	Anti-TNF + thiopurine + steroids	1 (3)	1 (3)	1 (3)	1 (3)	1 (3)	
	Anti-TNF + MTX	1 (3)	0	0	0	0	
	Thiopurine + 5-ASA	0	1 (3)	1 (3)	1 (3)	1 (3)	
	5-ASA + steroids	0	1 (3)	1 (3)	1 (3)	1 (3)	
IBD surgery (%)	Bowel resection	5 (15.2)					
	Perianal surgery	4 (12.1)					
	Pouch	1 (3)					
Flare (%)		4 (12.1)	6 (18.2)	5 (15.2)	3 (9.1)	2 (6.1)	.4446

CD: Crohn's disease; UC: ulcerative colitis; CD: standard deviation; IQR: interquartile range; y: years; IBD: inflammatory bowel disease; HBI: Harvey Bradshaw index; SCCAI: simple clinical colitis activity index; TNF: tumor necrosis factor; ASA: aminosalicylic acid

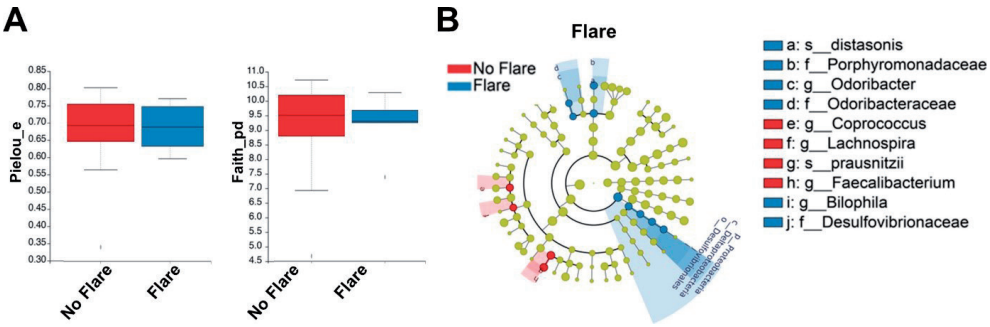


Supplementary Figure 1. Comparison of cytokine serum levels, C-reactive protein (CRP), estrogen and progesterone before, during and after pregnancy between for all IBD patients.

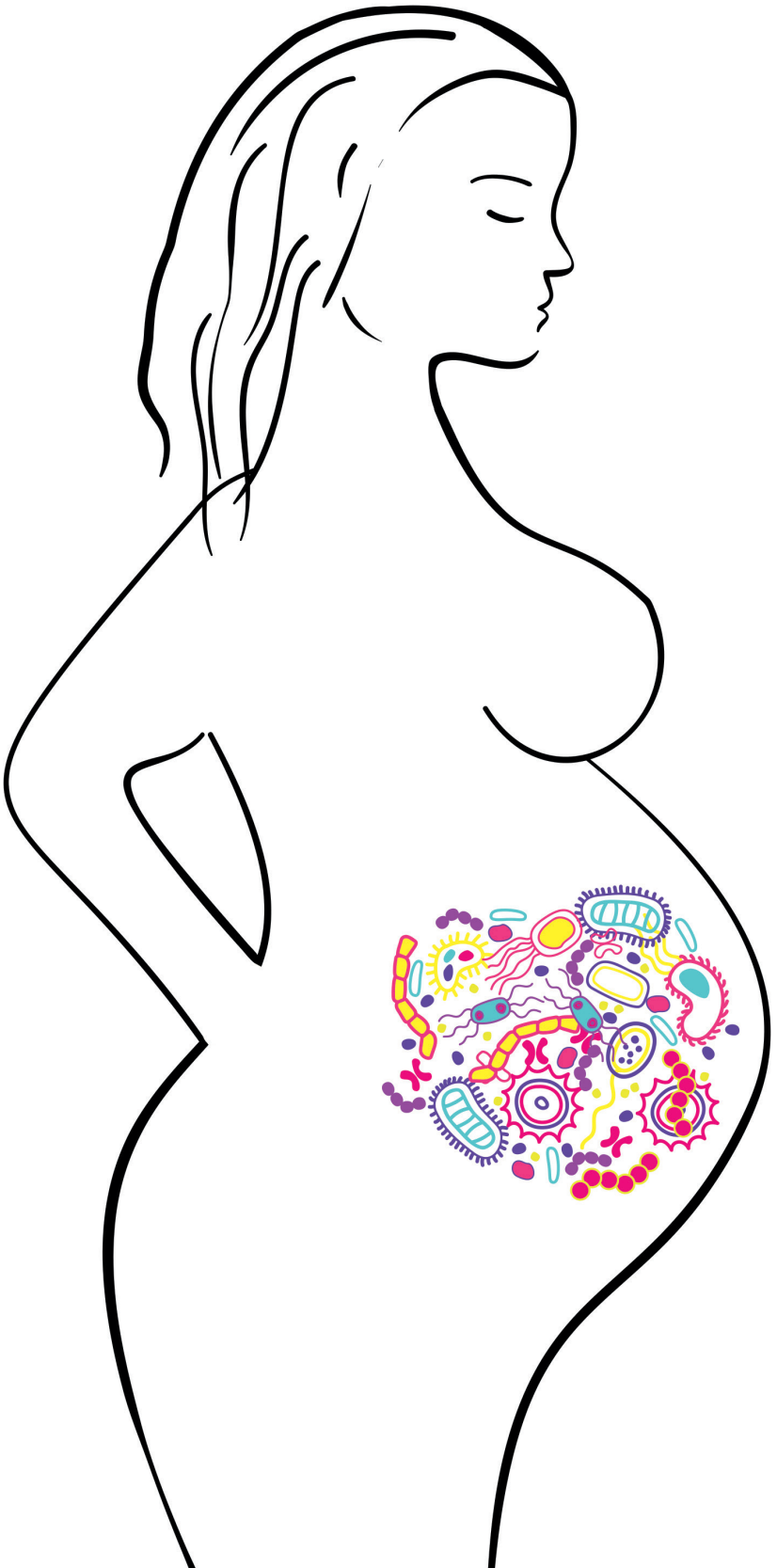


Supplementary Figure 2. Cytokine patterns in IBD patients are only modestly affected by treatment during pregnancy. Serum samples collected from IBD patients during gestation were divided according to the treatment received at that timepoint. (A) Number of patients on different medications during the three trimesters. Patients taking only anti-TNFα (TNF) medication, or in combination with other treatments (TNF+) were separated. (B) Principle coordinate analysis (PCA) shows no overall cytokine differences for patients on different medications. (C) Compared to untreated patients, patients on anti-TNFα single treatment (TNF) showed reduced IL-8 levels, patients on 5ASA showed reduced IL-10 and IL17 levels (*p<0.05, Mann Whitney U test).

Supplementary Figure 3. Cytokine patterns in IBD patients are patient-specific and stable during pregnancy. Heat map of cytokine profiles for IBD patients for whom paired samples of trimester 1 (T1), two (T2) and three (T3) were available. Patient samples clustering together are indicated by a colored square. Samples taken during inflammation are indicated with by an asterisk.

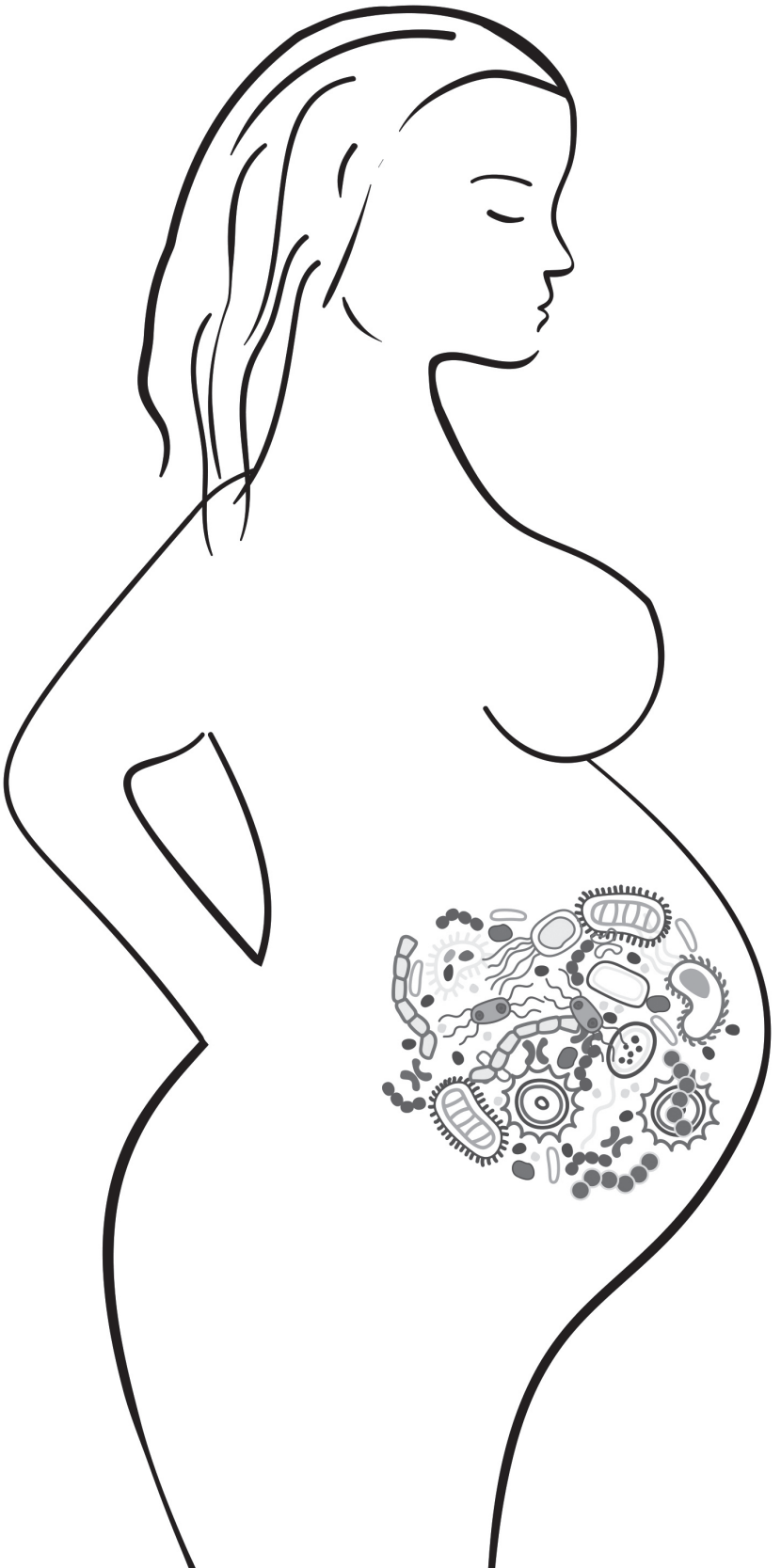


Supplementary Figure 4. Patients with UC experiencing a flare exhibited an increase in certain bacteria. Patients were divided according to those who suffered a flare (5 samples) during gestation compared to those with stable disease (19 samples). (A) Alpha diversity using Pielou's Evenness plot measurements and Faith's Phylogenetic Diversity; no significant differences were found. (B) Cladogram of significant differentially abundant microbial taxa obtained using LEfSe.



PART 2

BEDSIDE



CHAPTER 5

A first pregnancy seems associated with a positive effect on the course of inflammatory bowel disease: data from a prospective pregnancy cohort

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ABSTRACT

Objectives The effect of pregnancy on the course of inflammatory bowel disease (IBD) remains controversial. We aimed to describe the disease course before and after a first pregnancy in IBD patients.

Methods We analyzed data from a prospectively followed-up pregnancy cohort (minimal follow-up of 7 years), with clinical, biochemical and endoscopic characteristics obtained pre-pregnancy, during pregnancy and post-pregnancy. Possible factors associated with relapse (disease activity during pregnancy, maternal age, smoking, alcohol use, pre-pregnancy BMI, mode of delivery, thiopurine use during pregnancy, biological use during pregnancy, combination of thiopurine and biological use during pregnancy, breastfeeding, IBD diagnosis, endoscopic scores) were scored.

Results One hundred twenty six patients (95 Crohn's Disease [CD; 75%] and 31 Ulcerative Colitis/IBD unclassified [UC/IBD-U; 25%]) were enrolled, with one hundred pregnancies occurring in 100 primigravida patients. All pregnancies resulted in live birth. Twenty patients (20%) had a relapse during pregnancy. The median number of relapses/patient/year was 0.25 (IQR 0.5) and 0 (IQR 0.43) respectively before and after pregnancy (p=0.00). For CD patients the median relapses/person/year was 0.25 (IQR 0.5) before and 0 (IQR 0.25) after delivery (p=0.00), for UC/IBD-U patients there was no significant difference. In the post-partum period more UC patients relapsed compared to CD patients (68% vs 30.7%, p=0.01). Seven-year IBD-course was unchanged in the 26 women who did not become pregnant.

Conclusion In this prospective observational cohort study, we found a lower rate of relapses in the 4 years after delivery compared to the 3 years prior to a first pregnancy. Post-partum, more UC patients experienced a relapse compared to CD patients.

INTRODUCTION

IBD affects many women of childbearing age and gaining information on the effect of IBD on pregnancy and *vice versa* is of great importance, not only for a successful and healthy pregnancy, but also for the health of mother and child postpartum (1-3). Nevertheless, the effect of pregnancy on the course of Crohn's disease (CD) and ulcerative colitis (UC) remains controversial.

During pregnancy hormonal, immunological and microbial changes take place in the female body (4), which together allow for the growth of an MHC-mismatched fetus, suggesting an enhanced immunological tolerance. These changes are also observed in pregnant IBD patients. For instance, we showed that reduced microbial α-diversity present in IBD women at first trimester normalizes to diversity levels seen in healthy pregnancy at trimester 2 and 3 (5). Furthermore, serum pro-inflammatory cytokine patterns in IBD patients present pre-conception improve during pregnancy, and pregnancy hormones directly strengthen epithelial barrier functions (5,6). The observation that the microbiome from multiparous IBD women differs from that of nulliparous IBD patients suggests that these changes may have a lasting effect. Nevertheless, studies on the course of IBD during and after pregnancy are limited and contradictory. Pedersen and colleagues demonstrated in a prospective study that pregnant CD patients in remission prior and during pregnancy experienced a similar number of relapses when compared to matched non-pregnant patients. However, while not significant due to low patient numbers, post-partum remission seemed to be achieved more often for patients with active disease during the third trimester as compared to matched non-pregnant IBD women. For pregnant UC patients, a higher risk of relapse during pregnancy as well as post-partum was reported as compared to non-pregnant UC controls (7). In contrast, Castiglione *et al* described less relapses three years after childbirth compared to pre-pregnancy, for both UC and CD patients (8). A 10-year follow-up study described a decrease from 0.34 flares/year pre-pregnancy to 0.18 flares/year post-pregnancy in CD patients, while UC patients decreased from 0.76 flares/year pre-pregnancy to 0.12 flares/year post-pregnancy (9). In addition, it seems safe to stop anti-TNFα treatment in pregnant IBD patients without increasing the risk of a relapse during pregnancy (10). Of note, relapse rates are higher when CD patients conceive during active disease (7). For this reason, it is advised to strive for complete remission a minimum of 6 months prior to conception (11,12).

Thus, while molecular disease parameters appear to be beneficially modulated during pregnancy in IBD, controversy exists regarding their effect on clinical disease course. Therefore, the aim of our study was to assess the effect of pregnancy on the risk of relapse in primigravida IBD patients and to describe the disease course before and after a first pregnancy in patients with IBD.

METHOD

Study design and population

We analyzed data from our ongoing prospectively followed-up pregnancy cohort, where we collect clinical, biochemical and endoscopic characteristics prior to pregnancy, during pregnancy and post-partum. IBD patients with a pregnancy wish visited our outpatient clinic every 3 months when not pregnant, and every trimester during pregnancy. Patients were selected when the follow-up period was a minimum of 7 years and patients had not conceived before. When clinically necessary, patients were seen more frequently during this period. Not all included patients became pregnant during follow-up. To answer our main question we analyzed the course of the patients who did conceive separately. To assess if there is a difference between the women who did and did not conceive, we also describe the group in which there was no pregnancy.

Outcomes, data measurements and definitions

Relapse was defined as an endoscopic SES-CD score of ≥ 7 for CD, MAYO endoscopic score ≥ 2 for UC or Rutgeerts' score ≥ i1 and/or fecal calprotectin >200 µg/g and/or medication adjustment during follow-up. The SES-CD score is displayed as the SES-CD score divided by the number of segments obtained during endoscopy.

Statistical methods

Statistical analyses were performed using IBM SPPS (version 24.0 Chicago III, USA). Descriptive statistics of continuous variables are depicted as medians with interquartile range (IQR) or means with standard deviation (SD) and compared using T-tests or Mann Whitney U tests. Categorical variables are displayed in absolute numbers and percentages and compared using Chi-square or Fisher's exact tests. All tests were performed using 2-tailed tests. When the univariate analysis was significant we tested these factors in a multivariate analysis.

Ethical consideration

This study was approved by the ethics committee of the Erasmus Medical Center (Rotterdam, The Netherlands, MEC2013-579).

RESULTS

The study population

In total, 126 patients with IBD (95 CD, 28 UC and 3 IBD-unclassified [IBD-U]) were enrolled with a mean follow-up of 7.1±2.7 years. Of these, one hundred patients became pregnant during follow-up (75 CD, 23 UC and 2 IBD-unclassified [IBD-U]) and all pregnancies resulted

Table 1. Characteristics of pregnant and non-pregnant IBD patients

		Pregnant (n=100)	Non-pregnant (n=26)	p-value
Mean age (yrs, SD)		26.9 (4.2)	26.5 (5.6)	0.72
Diagnosis (%)	*Crohn's disease* *Ulcerative colitis* *IBD unclassified*	75 (75) 23 (23) 2 (2)	20 (76.9) 5 (19.2) 1 (3.8)	0.80
Median disease duration at start follow up (yrs, IQR)		3.5 (6)	5 (7)	0.41
Median follow up before pregnancy (yrs, IQR)		4 (3)	-	
Median follow up after pregnancy (yrs, IQR)		3 (2)	-	
Mean follow up (yrs, SD)		7.3 (2.9)	6.3 (1.7)	0.08
Live birth (yes,%)		100 (100)	-	
Smoking* (yes,%)		13 (13)	5 (19.2)	0.43
Smoking during pregnancy (yes,%)		6 (6)	-	
Mean BMI* (SD)		23.9 (4.2)	23.3 (4.3)	0.64
Mode of delivery (%)	*Vaginal* *Caesarean section*	61 (61) 39 (39)	-	
Breastfeeding (yes,%)		39 (39)	-	
IBD surgery* (%,yes)		35 (35)	7 (26.9)	0.44
Education level (%)	*Low* *Middle* *High*	5 (5) 49 (49) 46 (46)	3 (11.5) 13 (50) 10 (38.5)	0.44

*** In the case group this is preconceptional data**

in live birth. These women had a mean follow up of 7.3±2.9 years (3.5±1.9 years before pregnancy and 3.8±2.0 years post-partum) Twenty-six patients did not conceive. This group consisted of 20 CD patients (76.9%), 5 UC patients (19.2%) and 1 IBD-U patient (3.8%), with a mean follow-up time of 6.3±1.7 years falling within the same decade as the patients who did conceive. Baseline characteristics for the pregnant and non-pregnant patients are displayed in Table 1. Age was comparable among the groups (26.9±4.2 vs 26.5±5.6, P=0.72, respectively), as was educational level (p=0.44). Also BMI, smoking and whether patients underwent IBD surgery did not differ between pregnant and non-pregnant women.
Medication regimen of non-pregnant and pregnant patients is displayed in Figure 1.

Relapse per person per year

Overall, 74.6% of patients experienced a relapse during follow up (70.5% CD patients and 87.1% UC/IBD-U patients, p=0.06). In the time leading up to pregnancy, 68% of the UC/IBD-U patients experienced a relapse compared to 66.7% of the CD patients (p=0.902). Relapse during pregnancy occurred in significantly more UC/IBD-U patients (36%) compared to CD patients (14.7%, p=0.021). Within the 4 years post-partum, relapse also occurred more

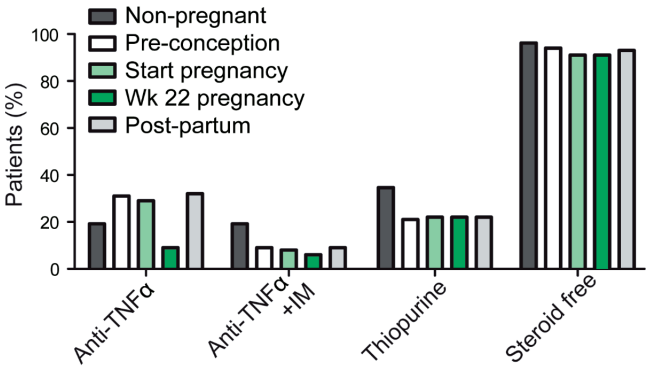

Figure 1. Medication regimen of non-pregnant and pregnant patients. For pregnant patients, pre-pregnancy, start of pregnancy, week 22 of pregnancy and post-partum medication use is indicated. Percentage of patients using anti-TNFα, using anti-TNFα plus immunomodulators (IM), using thiopurines and those that are steroid free are indicated.

often in the UC/IBD-U patients (68%) compared to the CD patients (30.7%, p=0.01). When comparing CD patients pre-pregnancy and post-partum, less patients showed relapses after giving birth (66.7% vs 30.7%, respectively, p=0.01, see Figure 2A). Consistent with the pre-pregnancy data of the conceiving patients, no differences were observed between CD and UC patients who did not become pregnant during the follow-up period.

The number of relapses/patient/year in the total IBD group was 0.25 (IQR 0.5) in the time leading up to pregnancy and 0 (IQR 0.43) in the years after (p=0.00). For CD patients (N=75) the median relapses/person/year was 0.25 (IQR 0.5) before pregnancy and 0 (IQR 0.25) after delivery (p=0.00). For UC/IBD-U patients (N=25) there was no significant difference in the median relapses/person/year before and after pregnancy (0.25 [IQR 1.0] vs 0.25 [IQR 0.5], p=0.57). The relapses/person/year during follow up time are displayed in Figure 2B, which shows that a decreasing trend in flare rate was already seen in the period leading up to pregnancy. When comparing the patients who became pregnant with those who did not, there was no significant difference in relapses/person/year in either the period prior to pregnancy (0.25 [IQR 0.5] for patients who became pregnant vs 0.18 [IQR 0.33] for those who did not, p=0.15) or the post-partum period of the IBD patients (0 [IQR 0.43]vs 0.18 [IQR 0.33], p=0.26, respectively).

Endoscopic scores

In total, 63 endoscopies were undergone by 45 patients in the 4 years prior to pregnancy, and 40 endoscopies were performed on 30 women in the 3 years post pregnancy. Endoscopies were performed for a suspected flare. For non-pregnant patients, 28 endoscopies were performed in 19 women during follow-up. Mean endoscopic SES-CD score for CD did not differ pre-conception compared to post-partum. Also when comparing post-partum with non-pregnant group there was no significant difference (see Figure 3A). MAYO score

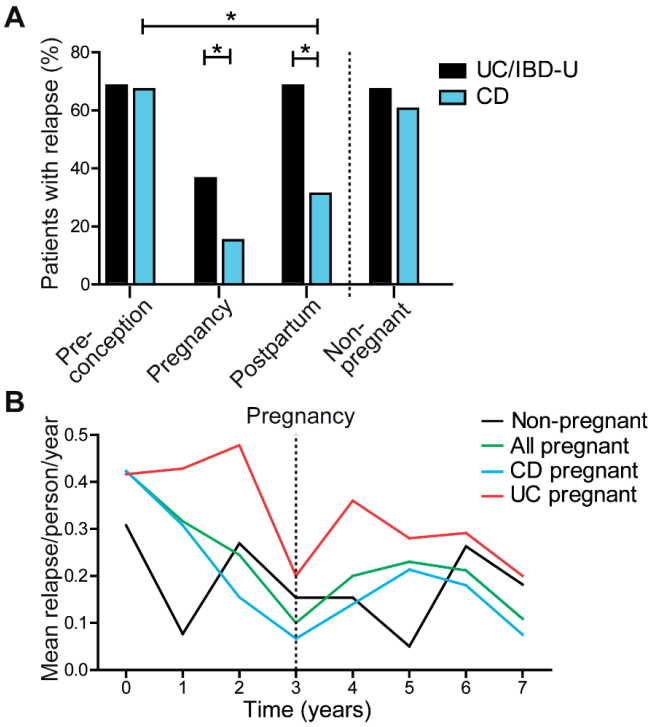

Figure 2. Disease course of IBD patients before, during and after pregnancy. (A) Significantly more patients with UC/IBD-U experienced relapses during pregnancy (36% vs 14.7%, respectively, p=0.021) and post-partum (68% vs 30.7%, respectively, p=0.01). Also, fewer CD patients presented with relapse post-partum as compared to pre-pregnancy (66.7% vs 30.7%, respectively, p=0.01). (B) Mean relapses per person per year during the complete follow up time. For the patients who became pregnant this is displayed as 3 years pre-pregnancy and 4 years after pregnancy.

of endoscopic severity of disease for UC also did not differ between pre-conceptional, post-partum and non-pregnant patients (see Figure 3B).

Possible patient-related factors associated with relapse rate

Factors as age, BMI, smoking, alcohol use, IBD related surgery and educational level did not significantly differ between patients who relapsed compared to those that did not. Similarly, mode of delivery and whether or not the patient had breastfed her child was not associated with relapsing post-partum.

In Figure 4A we display the percentage of patients on anti-TNFα therapy alone versus other IBD monotherapy experiencing a relapse per period. Patients who were on anti-TNFα post-partum seemed to have a better disease course (p=0.03). Patients who were on a combination of thiopurine and anti-TNFα also experienced less relapses than patients who were on another combination therapy, in particular in the post-partum period (p=0.01). For thiopurine therapy alone no significant difference was found (see Figure 4B and C).

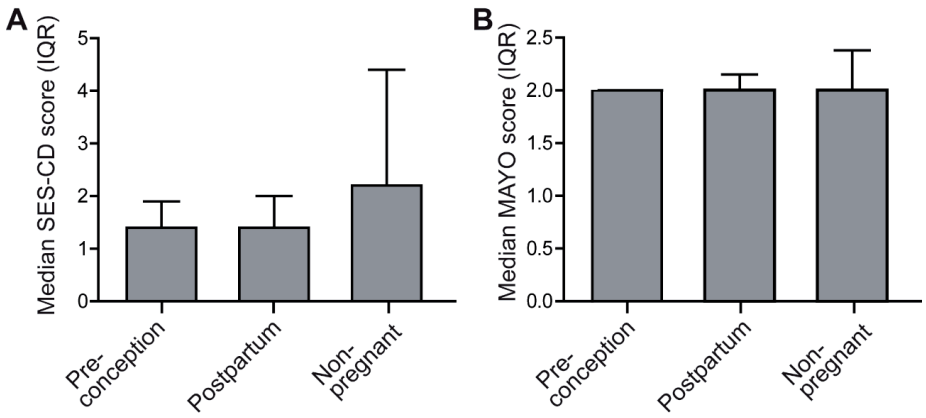

Figure 3. Endoscopy scores of IBD patients before and after pregnancy (A) Comparison of the median SES-CD endoscopy scores divided by segments obtained during endoscopy. No significant difference in score between preconception and post-partum was seen (1.4 [IQR, 0.5] vs 1.4 [IQR, 0.6], respectively, p=0.56). Furthermore no significant difference was found when post-partum score of pregnant patients was compared to non-pregnant patients (1.4 [IQR, 0.6] vs 2.2 [IQR, 2.2], respectively, p =0.15). (B) Comparison of MAYO scores for UC obtained during endoscopy. We did not find a significant difference in score between preconception and post-partum (2 [IQR, 0] vs 2 [IQR, 0.15], respectively, p=0.94). Also no significant difference was found when post-partum was compared to non-pregnant patients (2 [IQR, 0.15] vs 2 [IQR, 0.38], respectively, p=0.52).

To test if IBD diagnosis or the use of anti-TNFα treatment (single or in combination with thiopurine), both significant in univariate analysis, are independent predictors of relapsing post-partum, we performed logistic regression on these factors. For IBD diagnosis, the odds of experiencing a relapse post-partum are 88% higher in UC patients than CD patients (p=0.006). Anti-TNFα single treatment or in combination with thiopurine did not significantly affect the relapse rate in post-partum IBD patients.

DISCUSSION

In this prospective observational cohort study, we assessed the disease course before and after a first pregnancy in patients with IBD. We also describe the course of IBD in a group of women who did not conceive during follow up.

We found a lower number of relapses per person per year in the 4 years after delivery compared to the 3 years prior to a first pregnancy. Specifically, CD patients were less prone to experience a relapse after pregnancy than pre-pregnancy and we speculate that pregnancy has a positive influence on the course of disease in CD patients. Logistic regression identified UC patients more at risk for post-partum relapse compared to CD patients, while we also demonstrate that UC patients are more likely to relapse during pregnancy than CD

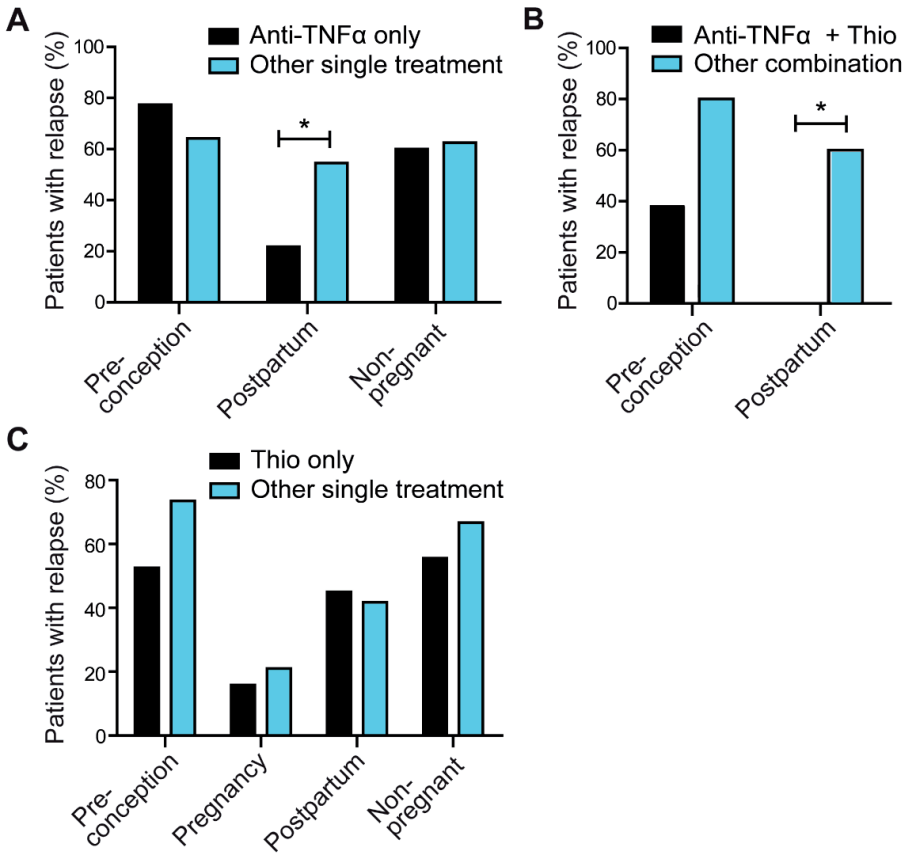

Figure 4. Relapse rate before, during and after pregnancy of IBD patients stratified by treatment regimen. (A) Comparison of anti-TNFα treatment only versus other monotherapy (thiopurine, systemic corticosteroids, 5-ASA or Naltrexone). Post-partum relapse rate was lower in patients using anti-TNFα. Data during pregnancy is not shown since in our center, women discontinue anti-TNFα use from week 22 onwards. (B) Patients using anti-TNFα and thiopurine as combination therapy post-partum experienced less relapses when compared to patients using other combination therapy (anti-TNFα and steroids, thiopurine and 5-ASA, thiopurine and steroids, 5-ASA and steroids). (C) Thiopurine use only did not affect relapse rate from IBD patients as compared to other single treatment.
Thio: Thiopurine.

patients. It has been speculated that differences in disease course between CD and UC during pregnancy might be explained by differences in the immunological pathways governing each disease. During pregnancy there is a shift towards a predominantly Th2 phenotype and therefore patients with Th2 dominant diseases, such as UC, might experience more relapses while those with Th1-driven disease, like CD, may benefit (4).
Unlike Pedersen et al., who showed a higher relapse rate of UC patients during pregnancy and post-partum compared to non-pregnant patients, our study did not reveal an increased

risk of flaring of UC patients compared to pre-pregnancy or non-pregnant controls (7). This discrepancy may potentially be explained by the longer follow-up time of 4 years in our study, in contrast to the 6 months of Pedersen et al.

Another contributing factor to the lower relapse rates could be adequate preconceptional counseling. Over the last years the importance of pregnancy counseling for women with IBD has gained attention. Adequate counseling resulted in less relapses during pregnancy, mainly due to drug adherence during pregnancy (1). Indeed our current study indicates that relapse rates already decline prior to conception. It is advised to strive for a sustained remission of at least 6 months prior to conception and it is conceivable that this may partly be responsible for a more stable disease from pregnancy onwards (11,12). However, it should be noted that non-pregnant IBD patients, who received the same care and counseling, did not show a reduction in relapses/person/year in this study. Furthermore, the effect was more pronounced for pregnant CD patients than for pregnant UC patients, suggesting that biological differences account for at least part of this effect. For future studies it would be informative to describe the effect of optimization of IBD medication prior to pregnancy on relapse rates.

Over the last years there is less reluctance to actively treat IBD patients during pregnancy. For instance, in this study 79% of the patients received IBD-related medication during pregnancy, compared to 81% before pregnancy. The exception are biologicals, treatment of which is often ceased at the end of second trimester. We showed a better disease course after pregnancy in patients using anti-TNF-α (with or without thiopurine). Patients restarted their anti-TNF-α within six weeks after delivery in a same dose as used previously and therefore an 'induction therapy' effect is not likely to contribute to the lower relapse rate. In addition to our findings, Rottenstreich et al. found that biologic therapy is an independent protective factor against relapse already during pregnancy (13). This study has several strengths. First, the IBD patients were followed for a mean period of 7.1 years. In this period the patients visited the outpatient clinic on a regular and comparable basis. All patients received the same care and counseling. Secondly, our pregnant and non-pregnant groups were followed in the same era, as development of knowledge around medication is changing quickly. As shown in Table 1, pregnant and non-pregnant patients in our group were comparable. In our study we also took severity of relapse into account by including endoscopy scores.

This study also has some limitations that need to be addressed. First, our cohort consists of a tertiary patient population who received specialized care during their visits to the outpatient clinic. However, over the last years this approach has become standard of care in many hospitals. Secondly, we designed a prospective observational cohort study and therefore were not able to match the cases and controls described in our study, which may lead to confounding. To eliminate confounding effects, a matched case-control study needs to be conducted. Thirdly, we did not perform a sample size calculation prior to the study

and our study populations might be underpowered to make comparisons between groups, however the aim of our study was to describe the course of IBD around pregnancy in our total group.

In conclusion, these data are in favor of pregnancy having an effect on disease course, with in particular CD patients showing a natural decrease in disease activity over time. Larger case-control studies of pregnant and not pregnant women are needed to further investigate the course of IBD and its molecular consequences.

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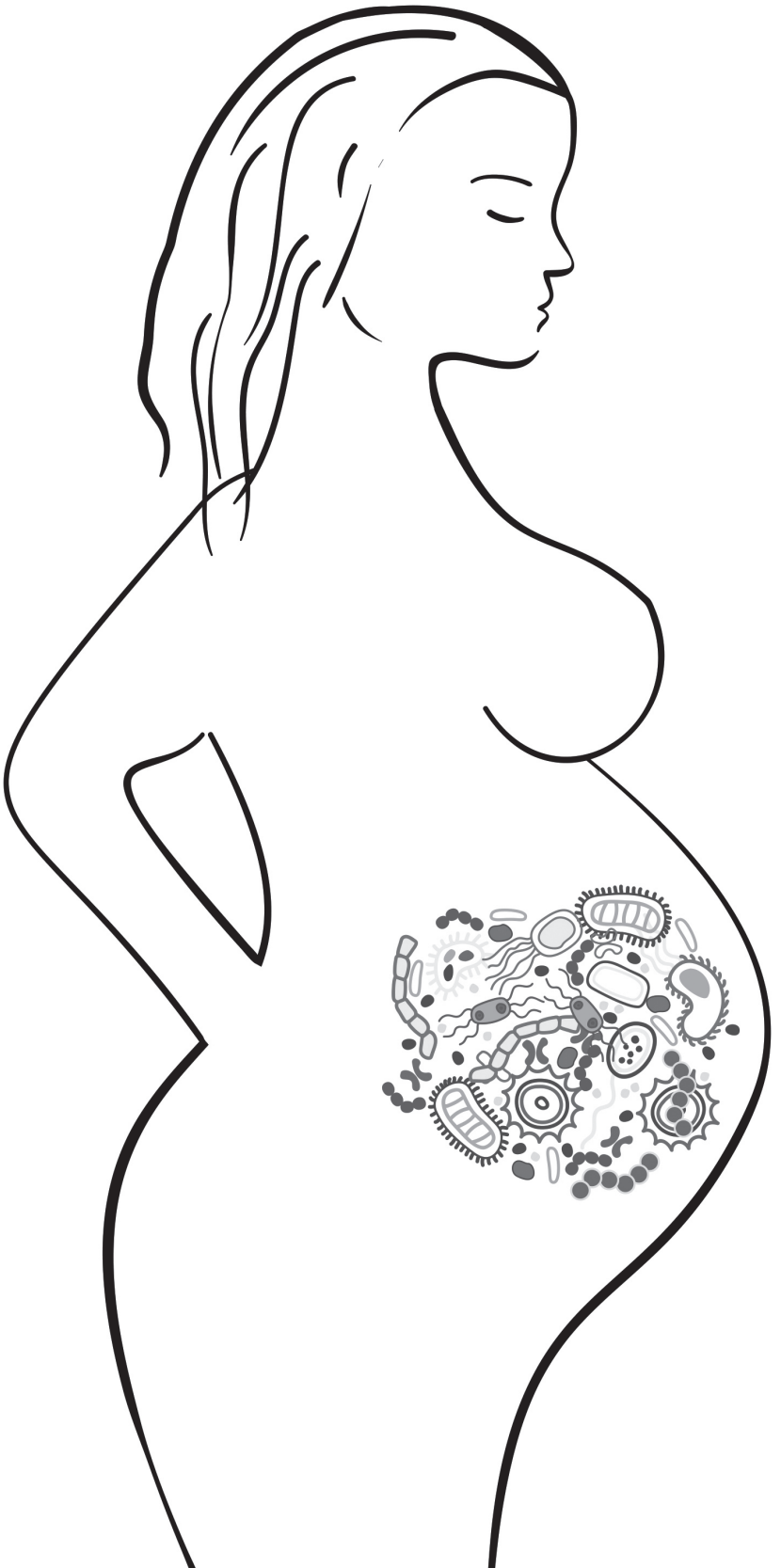

CHAPTER 6

Health-related quality of life in the first 5 years of the children born to mothers with IBD does not differ from children born to healthy mothers

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ABSTRACT

Objective Inflammatory bowel disease (IBD) is often diagnosed in women in their reproductive years of life and therefore children are born to mothers with IBD. Health outcomes of children born to mothers with IBD seem favorable. However, little is known about the quality of life related to their health compared to children born to healthy mothers. Therefore, our first objective was to investigate the effect of having IBD during pregnancy on the health-related quality of life (HRQoL) of children born to mothers with IBD in the first 5 years of age compared to children born to healthy mothers. Secondly, we studied the effect of the different IBD related factors on the HRQoL.

Methods We prospectively followed 264 women with IBD, who visited the preconception outpatient clinic at our tertiary health center in the Netherlands from April 2013 through November 2016. Mothers of children aged 1-5 years were approached to fill in a 43-item validated TNO-AZL Preschool Children Quality of Life questionnaire (TAPQOL) to assess HRQoL (1,2). Outcomes were compared to children of mothers without IBD.

Results One-hundred-eighty-two women completed the TAPQOL questionnaire. In total 182 children of mothers with IBD were included [median age 3.0 years (IQR 2-4)]. From 70 healthy mothers, 70 children were included as controls. There was no significant difference in the HRQoL between children who were and were not born to mothers with IBD (P=0.18). Also, no effect of the different IBD-related factors was found.

Conclusion In this study, we found no effect of having IBD during pregnancy on the health-related quality of life of children in the first 5 years of life.

INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic inflammation of the digestive tract, consisting of Crohn's disease (CD), ulcerative colitis (UC) and IBD-unclassified. The incidence and prevalence increased over the last years, with the mean incidence peak between 15-25 years (3,4). Therefore, it affects women in their reproductive years and children are born to these mothers with IBD.

Most studies, regarding pregnancy and IBD, focused on the clinical health outcomes of children and these studies are reassuring with regards to health outcomes such as congenital malformations and first year infectious diseases in children born to mothers with IBD (5). However, little is known about the effect of having IBD during pregnancy on the quality of life of offspring. Quality of life is not only determined by clinical health outcomes, but also by mental and psychological factors. Data on health-related quality of life (HRQoL) are needed for adequate preconceptional counseling, as studies show a higher voluntary childlessness in women with IBD compared to healthy women (6) and women with IBD breastfeed their baby for a shorter period compared to the general population (7). The main reason for this is poor knowledge and anxiety of pregnancy-related issues in IBD (8). Moreover, when offered IBD-specific reproductive knowledge the odds of voluntary childlessness for IBD women is lowered (9).

Sparse publications exist on the psychological health outcomes and quality of life of the children born to mothers with IBD. In a small cohort of 30 children exposed to intrauterine thiopurines, HRQoL did not differ from an historical control group (10). Of special interest in this context is recently published data that showed a new onset of mental disorders in a large population-based Canadian cohort in females suffering from IBD, especially in the first year after giving birth (11). This might affect the child's emotions and quality of life.

To optimize our preconception counseling we aimed to assess the HRQoL of children (1-5 years) born to mothers with IBD from our prospectively followed up pregnancy cohort and compare these outcomes to children born to healthy mothers. As HRQoL is determined by clinical health outcomes as well as psychological and mental factors, we analyzed the effect of IBD subtype, IBD medication, IBD surgery prior to pregnancy, disease activity during pregnancy, whether infants were breast or formula fed, prematurity, the rate of infections, allergies, congenital abnormalities and the child's gender and combined these data with a validated HRQoL questionnaire in the study group.

METHODS

Study design and population

All women who visited the IBD pregnancy outpatient clinic at the Erasmus Medical Center from April 2013 through November 2016 and gave live birth were invited to participate

and to fill in the TAPQOL questionnaire concerning their child(ren) aged 1-5 years ("Study group"). All females at the Erasmus MC diagnosed with IBD receive active preconception counseling on different aspects of the influence of IBD and medication on the outcomes of their babies such as the use of medication before and during pregnancy, the effect of pregnancy on disease, the effect of disease on the fetus and the potential passing on of IBD to their offspring. Patients also receive information about the risk of active IBD on pregnancy complications such as preterm birth or low birth weight (12). Inclusion criteria were as follows: children aged 1-5 years, ability of the parent to understand a Dutch questionnaire and a mother diagnosed with IBD prior to and during pregnancy. Mothers were approached via e-mail, phone and/or by mail. Additionally, Information from the mothers on the child's gender, age, growth, allergies, national program vaccinations, whether infants were breast or formula fed, prematurity, infections and whether hospital admission was necessary for infection was obtained. Data on maternal age, education level, IBD subtype, IBD surgery prior to pregnancy, IBD medication, and disease activity during pregnancy was collected from the prospective pregnancy database. We questioned whether the historical control group, consisting of 340 children from the same geographic region as the study group, of the original TAPQOL study (1) would still be comparable to the current healthy children. For example there has been an increase in atopic sensitization rate among Dutch children and a higher rate of allergic diseases among these children (13). Therefore a new control group, consisting of children from healthy mothers, was formed ("Control group"). We compared the outcomes of this group to our study group. Mothers of these children were friends or relatives of mothers with IBD. Inclusion criteria for this control group were as follows: children aged 1-5 years, ability of the parent to understand a Dutch questionnaire and a mother reported to have no underlying mental or physical health issue and no use of medication.

Outcomes, data measurements and definitions

For the assessment of HRQoL of these children, the validated TNO-AZL Preschool Children Quality of Life Questionnaire (TAPQOL) was used (1,2). This 43-item questionnaire is designed for preschool children aged 1-5 years old. It is a validated multidimensional generic instrument that is based on parental reporting and reflects the child's HRQoL in the last three months.

The TAPQOL consists of 12 scales that cover four main domains: physical functioning (sleeping, appetite, lung problems, stomach problems, skin problems, motor functioning), social functioning (problem behavior), cognitive functioning (communication) and emotional functioning (positive mood, anxiety, liveliness).

The TAPQOL-scores were converted into a 0-100 scale, using a syntax file provided by the authors of the TAPQOL. The results are presented as total functioning and domain functioning scores. The total TAPQOL score is calculated by averaging the 12 scales. A higher score indicates a better quality of life.

Maternal IBD disease activity during pregnancy was defined as: a Harvey Bradshaw Index (HBI) for CD>5 and Simplified Clinical Colitis Activity Index (SCCAI) for UC > 2, and/or either C-reactive protein (CRP) >9.0 mg/l or fecal calprotectin >200 µg/g.
It has been shown that there is a difference in infection rate during the first years of life (14), with more infections reported in the first 2 years of life compared to pre-school age (3-5 years). For this reason, an age group from 0-2 years was formed in order to describe infection rate.

Statistical methods

Statistical analysis was performed using IBM SPPS (version 24.0 Chicago III, USA). Descriptive statistics of continuous variables are depicted as medians with interquartile range (IQR) or means with standard error of the mean (SEM) and compared using T-tests or Mann Whitney U tests. Categorical variables are displayed in absolute numbers and percentages and compared using Chi-square or Fisher's exact tests. All tests were performed using 2-tailed tests. To compensate for multiple testing, a P-value of <0.004 was considered significant. The effect of IBD subtype, maternal IBD medication use, IBD surgery prior to pregnancy, disease activity during pregnancy, breastfeeding, prematurity, the occurrence of infections, allergies, congenital abnormalities, age of the mother and the child's gender on the total TAPQOL score were analyzed in a univariate analysis. When the univariate analysis was significant (P-value of < 0.05) a multivariate analysis was performed.

Ethical consideration

This study was approved by the ethics committee of the Erasmus Medical Center (Rotterdam, The Netherlands).

RESULTS

The study population

In total, 259 mothers with IBD and 272 children were asked to participate. One hundred eighty-two mothers (70.3%) participated and questionnaires concerning 182 children were completed and returned. The control group we used consisted of 70 children of 70 healthy women. Baseline characteristics of the 340 children in the historical control group compared to our study group are shown in Supplementary Table 1**.**

Mothers

Maternal characteristics are displayed in Table 1. Age was comparable among the study and control group (35.1 ±4 vs 34.3±3.4, P=0.1, respectively). In the IBD group the proportion of mothers diagnosed with Crohn's disease was slightly higher (57%, n=104) than mothers diagnosed with ulcerative colitis or IBD unclassified (IBD-U). Education level of mothers

Table 1. Characteristics of mothers with and without IBD

		Cases (n=182)	Controls (n=70)	p-value
Mean age (yrs, SD)		35.1 (4.0)	34.3 (3.4)	0.1
Diagnosis (%)	*Crohn's disease* *Ulcerative colitis* *IBD unclassified*	104 (57) 70 (38.5) 8 (4.4)		
IBD maintenance therapy (Monotherapy, %)	*no medication* *anti-TNF* *thiopurine* *systemic corticosteroids* *5-ASA*	36 (19.8) 50 (27.5) 36 (19.8) 13 (7.1) 18 (9.9)		
IBD maintenance therapy (Combination, %)	*anti-TNF and thiopurine* *5-ASA and steroids* *5-ASA and thiopurine*	19 (10.4) 5 (2.7) 5 (2.7)		
IBD surgery prior to pregnancy (%)		5 (2.7)		
Number of children (%)	*1* *2*	164 (90.1) 18 (9.9)	67 (95.7) 3 (4.3)	0.15
Education level (%)	*Low* *Middle* *High*	1 (0.5) 76 (41.8) 105 (57.7)	0 (0) 21 (30) 49 (70)	0.18

was comparable in both groups (P=0.18) and the majority was highly educated, meaning they obtained a bachelor or master's degree (57.7% in the study group versus 70% in the control group).

Child demographics and congenital abnormalities

Median age of the children of women with IBD was comparable with the median age in the control group (3 yrs [IQR 2-4] vs 3 yrs [IQR 2-4], P=0.55, respectively), as was gender (51.1% of children were female in case group vs 58.6% of the controls, P=0.29) and parent-reported growth (95.6% normal length for age in case group vs 100% normal length for age in control group, P=0.12), see Table 2. In the study group 4 (2.2%) children had a congenital abnormality (ventricular septum defect, transposition of the great vessels, polydactyly, congenital hydronephrosis), in the control group no congenital abnormalities were reported (P=0.52).

The children in the historical control group were younger than our study group children (2.5 yrs [IQR 1.8-3.3] vs 3 yrs [IQR 2-4], P<0.001, respectively).

Infectious diseases, allergies and vaccination rate

We found no differences in parental reported outcomes in the IBD group vs controls (Table 3). In the study group 92 (50.6%) children experienced an infection during follow up of whom 53 (29.1%) experienced more than one infection. In the control group 30 (42.9%) children experienced an infection of whom 20 (28.6%) more than one infection. This

Table 2. Characteristics of the children

		Cases (n=182)	Controls (n=70)	p-value
Median age (yrs, IQR)		3 (2-4)	3 (2-4)	0.55
Age group	*Infants, 0-2 yrs (%)*	12 (6.6)	10 (14.3)	0.1
Gender (% female)		93 (51.1)	41 (58.6)	0.29
Growth (% normal)		174 (95.6)	70 (100)	0.12
Preterm birth (% yes)		13 (7.1)	4 (5.7)	0.69
Congenital abnormalities (% yes)		4 (2.2)	0 (0)	0.52

Table 3. Reported infections, allergies and vaccination of the children

		Cases (n=182)	Controls (n=70)	p-value
Median parental reported infections per person per yr (IQR)	*Total group* *Infants (0-2 yrs)*	0 (0-1) 0.5 (0-1.75)	0 (0-1) 0 (0-3.5)	0.92 0.87
Infection related hospitalization, N (% yes)		7 (3.8)	2 (2.9)	0.71
Allergies, N (% yes)		19 (10.4)	8 (11.4)	0.91
Vaccination (participation in national program), N (% yes)		177 (97.3)	68 (97.1)	0.96

accounted for a median parental reported infections per person per year of 0 (IQR 0-0.1) in the study group versus 0 (IQR 0-0.1) in the control group (P=0.92). Seven (3.8%) children in the study group and 2 (2.9%) in the control group were admitted to the hospital because of an infection (P=0.71).

As explained above we formed an age group from 0-2 years. In children aged 0-2 years, 6 (50%) of the 12 study children experienced an infection and 3 of them had more than one infection versus 4 (40%) of the 10 children in the control group. The median parental reported infections per person per year in this age group was 0.5 (IQR 0-1.75) in the study group and 0 (IQR 0-3.5) in the control group (P=0.87). The number of children with allergies, as reported by parents, was comparable between cases and controls (10.4% vs 11.4%, P=0.91, respectively). Also, no differences in vaccination rate were found (97,3% vs 97.1%, P=0.96).

In contrast with our own control group, in the historical control group there was a difference in reported allergies compared to the study group (3.5% versus 10.4%, respectively, P=0.001).

TAPQOL 12 domains

In all the 12 domains (stomach, dermatological, pulmonary, appetite, energy level, mood, problem behavior, anxiety, social, sleeping, motor functioning and communication) no difference was found between infants of mothers with IBD and controls (Table 4).

Table 4. Median (IQR) scores on 12 domains of the TAPQOL questionnaire

	Cases (n=182)	Controls (n=70)	p-value
Stomach	91.7 (83.3-100)	87.5 (75-100)	0.04
Skin	91.7 (83.3-100)	91.7 (83.3-100)	0.86
Lung	100 (83.3-100)	100 (91.7-100)	0.52
Sleep	87.5 (75-100)	81.3 (75-93.8)	0.15
Appetite	91.7 (75-100)	87.5 (75-100)	0.4
Liveliness	100 (100-100)	100 (100-100)	0.24
Positive mood	100 (100-100)	100 (100-100)	0.33
Problem behaviour	71.4 (64.3-83.9)	64.3 (64.3-78.6)	0.22
Anxiety	83.3 (66.7-100)	83.3 (50-100)	0.42
Social functioning	100 (100-100)	100 (100-100)	0.24
Motor functioning	100 (100-100)	100 (100-100)	0.42
Communication	100 (93.8-100)	100 (93.8-100)	0.52
Total score	90 (86.5-93.3)	89.1 (84.4-92.3)	0.18

Table 5. Univariate analysis of possible factors associated with altered HRQoL score in IBD group

	N (%)	p-value
Diagnosis (CD/UC/IBDU)	104 (57.1)/70 (38.5) /8 (4.4)	0.99
IBD medication (yes/no)	146 (80.2)/36 (19.8)	0.24
IBD bowel surgery prior to pregnancy (yes/no)	53 (29.1)/129 (70.9)	0.39
Disease activity during pregnancy (yes/no)	49 (26.9)/133 (73.1)	0.96
Breastfed (yes/no/unknown)	67 (36.8)/108 (59.3)/7 (3.8)	0.17
Premature (yes/no)	13 (7.1)/169 (92.9)	0.73
Gender child (f/m)	93 (51.1)/89 (48.9)	0.57
Infections (yes/no)	92 (50.5)/90 (49.5)	0.64
Allergies (yes/no)	27 (14.8)/155 (85.2)	0.2
Congenital abnormalities (yes/no)	4 (2.2)/178 (97.8)	0.28

HRQoL

The 12 domains as described are considered essential for 1-5-year-old children. The average of these domains is the HRQoL. The overall HRQoL did not differ between the study and control group (90 [IQR 86.5-93.3] vs 89.1 [IQR 84.4-92.3], respectively, P=0.18).

IBD-related factors affecting HRQoL

In the study group, no association was found between the HRQoL and IBD subtype (P=0.99), IBD medication (P=0.24), IBD surgery prior to pregnancy (P=0.39), disease activity during pregnancy (P=0.96), whether infants were breast or formula fed (P=0.17), prematurity (P=0.73), the child's gender (P=0.57), rate of infections (P=0.64), allergies (P=0.2) or congenital abnormalities (P=0.28), see Table 5.We also found no association between the HRQoL and the different types of IBD medication (Supplementary Table 2).

DISCUSSION

In this study, we assessed the effect of IBD on the health-related quality of life of children aged 1-5 years.

We show that the HRQoL of children born to mothers with IBD is comparable to the HRQoL of children born to mothers without IBD. This is reassuring and implies that there are no major effects of maternal IBD on the quality of life of their offspring, as reported by the parents.

A previous study reported that thiopurine use during pregnancy and lactation in women with IBD did not affect the HRQoL of their children up to 6 years of age (10). We were able to confirm these results in a larger and more heterogeneous group of children, and we found that IBD and the use of different IBD drugs during pregnancy had no effect on the HRQoL of the child.

A higher HRQoL score, although not significant, in children of mothers with IBD compared to the children in the control group was seen in the stomach domain. It could be hypothesized that women with IBD are more experienced with gastrointestinal problems leading to a different evaluation. However, it should be noted that the reliability of the stomach functioning scale in the TAPQOL questionnaire was reported low and therefore results should be taken with caution and might not be of relevance (1).

As many mother-related factors may affect the HRQoL of children, we were also interested in the effect of these factors on the HRQoL. However, IBD subtype, IBD surgery prior to pregnancy, disease activity during pregnancy do not affect the quality of life of the children. Also, factors related to the child, such as prematurity, the occurrence of infections, allergies, congenital abnormalities and the child's gender do not affect the subjective reported quality of life of the children, as measured by TAPQOL questionnaire in our cohort. Of interest is also whether infants were breast or formula fed, which reassuringly showed no difference in feeling of wellbeing in the children.

Earlier studies that reported HRQoL in children with congenital abnormalities and prematurity reported a lower HRQOL in these children (15–17). However, literature mainly reported on objective determinants related to quality of life, as health status and functioning. Also, it should be noted that our subgroups have a low number of patients, and more premature children and children with congenital abnormalities need to be included to draw conclusions. Congenital abnormalities were reported in our study group, although it did not occur statistically more than in our control group. This confirms results from previous studies that IBD and IBD medication use during pregnancy have no influence on congenital abnormalities (18–20). To maintain remission, females are advised to continue their medication, such as biologicals and thiopurines, during pregnancy. It is reassuring that women who continue these drugs during pregnancy do not have a higher risk of adverse pregnancy outcomes, such as congenital abnormalities, preterm delivery or low birth weight (18–20). Although a previous study reported an increased infection risk in children exposed *in utero* to a com-

bination of anti-TNFα therapy and thiopurines compared to children exposed to anti-TNFα monotherapy (21), we and others have shown that treatment with immunosuppressive IBD medication during pregnancy is not associated with increased rate of serious infections requiring hospital admission of children in the first years of life (22,23). Furthermore we have found no association with adverse reactions to vaccination, growth failure, auto-immune diseases and malignancies in children until 5 years of age (5). We did report a higher rate of allergies in our study group than the historical control group, but the rate of allergies between the study group and our newly formed control group was comparable. The comparison between our control group and the historical control group confirms the previous described increase in atopic sensitization rate among Dutch children and a higher rate of allergic diseases among these children (13).

This study has some limitations that need to be addressed. The TAPQOL questionnaires are based on self-reported data and cannot be independently verified. Therefore, it can contain potential sources of bias such as selective memory, attribution and exaggeration. In our study, parents recalled a mean number of 0 infection episodes per year, whereas other studies have reported higher rate of infections in the general population: mean annual number of 3.4 episodes of infection in infancy and 2.3 episodes in preschool children (14).

Previously it was described that stress due to an illness of the parent also determines the quality of life in children (24). We found no differences between children of mothers with IBD and children born to healthy mothers, but we did not have information on the course of the IBD during the 3 months reported on in the TAPQOL questionnaire and therefore we cannot conclude on the effect of active IBD on the mother.

Future research should take the mental health of the mothers on the HRQoL of children into account, as recently published data showed a new onset of mental disorders in a large population based Canadian cohort in females suffering from IBD, especially in the first year after giving birth (11). Adding emotional functioning as a variable could affect outcomes of HRQoL and might differ within age groups. Further research such as a case-control study is needed. Finally, the TAPQOL is designed to evaluate the HRQoL of a child at a given point in time. Consequently, a follow-up would be necessary in order to acquire a more dynamic view of the child's HRQoL.

CONCLUSION

The health-related quality of life in children born to mothers with IBD does not differ from the health-related quality of life of children born to healthy mothers. IBD related factors do not seem to influence the quality of life of their children. The results are reassuring and can be used to further strengthen the preconceptional counseling.

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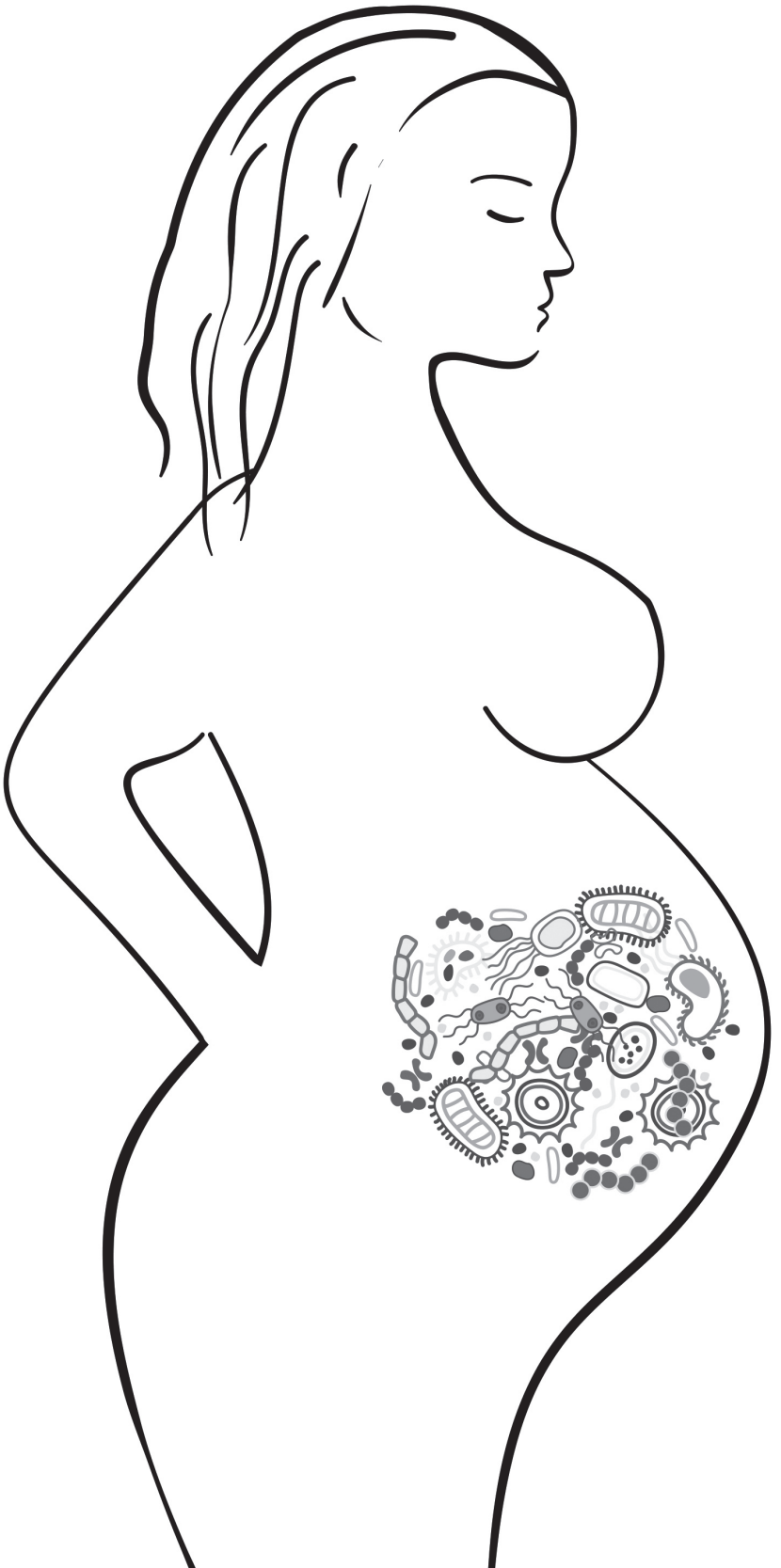
SUPPLEMENTARY MATERIALS

Supplementary Table 1. Baseline characteristics children with historical control group

	Cases (n=182)	Historical Controls (n=340)	p-value
Median age (yrs, IQR)	3 (2-4)	2.5 (1.8-3.3)	<0.001
Gender (% female)	93 (50)	146 (42.9)	0.09
Growth (% normal)	176 (96.7)	327 (96.2)	0.17
Allergies (%)	19 (10.4)	12 (3.5)	0.001

Supplementary Table 2. Univariate analysis of HRQoL score in relation to different IBD medication

	N (%)	p-value
anti-TNF (yes/no)	54 (27)/42 (21)	0.93
thiopurine (yes/no)	38 (19)/42 (21)	0.56
systemic corticosteroids (yes/no)	15 (7.5)/42 (21)	0.16
5-ASA (yes/no)	20 (10)/42 (21)	0.17
anti-TNF and thiopurine (yes/no)	19 (9.5)/42 (21)	0.7
5-ASA and steroids (yes/no)	6 (3)/42 (21)	0.55
5-ASA and thiopurine (yes/no)	6 (3)/42 (21)	0.42



CHAPTER 7

Summary and general discussion

This thesis aimed to extend our knowledge regarding IBD and reproduction from bench to bedside.
IBD is frequently diagnosed in women's reproductive years of life and concerns arise on how IBD affects their pregnancy and how pregnancy affects the course of IBD. A recent study described the association between moderate to severe IBD and lower live birth rates, more spontaneous abortions and caesarean sections (1). Therefore it is important to broaden our knowledge regarding pregnancy-related physiology in IBD patients and strive for a healthy pregnancy. Gaining a better insight into the physiology and pathology of both pregnancy and IBD may in the long run allow us to predict which patients may experience reduced or increased disease activity during pregnancy and post-partum.
In the first part of this thesis we described the molecular basis, the microbial and hormonal changes, the effect on epithelial barrier and the inflammatory system that take place during pregnancy in IBD patients. The second part focusses on the clinical outcome of the changes described in the first part, also taking into account the potential effect on the quality of life of children born to these mothers.

PART I BENCH

Pregnancy is a unique state requiring hormone-induced changes and modulation of immune function. In CHAPTER 2 we reviewed these pregnancy-induced changes and their potential effect on the course of IBD. We discuss the general consensus regarding the immunology of pregnancy. Limited recognition and response to fetal cells both contribute to fetal tolerance during pregnancy and allow a MHC-mismatched fetus to grow. In addition, the response of immune cells is also modulated during pregnancy, with a pro-inflammatory Th1 cytokine pattern during implantation followed by a shift toward a Th2-phenotype for the main duration of pregnancy and again a Th1-phenotype during labor. A recent study confirmed this switch from pro-inflammatory toward anti-inflammatory upon conception in women who conceived after embryonal transfer. In this study, they found that women who conceived after embryonal transfer had an increase in pro-inflammatory peripheral cytokines upon conception and a switch to an anti-inflammatory cytokine profile during implantation. Women who did not conceive after embryonal transfer did not switch toward an anti-inflammatory cytokine change (2). This broad shift towards a Th2 phenotype for the largest part of the pregnancy period is speculated to be in favor of Th1-mediated diseases such as CD. However to what extent these local changes reflect peripheral effects remains less clear. In CHAPTER 4 we describe that peripheral blood cytokine levels in IBD patients change significantly and beneficially from pre-conception to pregnancy, suggesting that pregnancy may indeed positively affect disease characteristics. For the immunological changes we showed decrease of pro-inflammatory cytokines (IL-6, IL-8, IL-12, IL-17 and TNF-alfa) upon conception, while during pregnancy cytokine levels remained stable. However,

we did not observe a specific Th1 or Th2 shift, nor did we distinguish differences between CD and UC patients in this study. Regulatory T-cells, another T-cell subset, also contribute to tolerance during pregnancy by suppressing T-cell activation. Pregnancy hormones, like human chorionic gonadotropin (hCG), mediate early expansion of these regulatory T cells. Since the publication of our review, a new study provided additional insight into the effect of estrogen, which also increases during pregnancy, on regulatory T cell modulation in the inflamed intestine (3). Goodmana et al. showed that estrogen signaling on ER-beta receptors is required for differentiation of TGF-beta-dependent expansion of Tregs in the gut and their immunoprotective role and we speculate that this is one way in which estrogen could contribute to intestinal immune tolerance during pregnancy in IBD.

During pregnancy sexhormones estrogen and progesterone increase, until the third trimester. Studies suggested anti-inflammatory effects of sexhormones by reducing inflammatory cytokines (4–6). Clinical studies on the effect of these hormones on the course of IBD are conflicting, as are animal studies (7–12). Furthermore animal studies are difficult to translate to pregnant IBD patients and the direct effect of these hormones stayed unclear. Therefore, in CHAPTER 3 we examined the direct effect of estrogen and progesterone on intestinal epithelial barrier cells and their function. The epithelial barrier is the first line of defense against invading microorganisms. In IBD patients, immune cells produce inflammatory cytokines, such as IFN-gamma and TNF-alpha, causing a process referred to as the 'leaky gut'. This weakened intestinal barrier function is an important aspect of IBD pathophysiology (13,14). During active disease there is additional reduction in tight junctions which impact on the regulation of epithelial permeability (15). Furthermore, there is an accumulation of unfolded protein inside the endoplasmic reticulum due to inflammatory triggers impeding protein folding in IBD. This is referred to as endoplasmic reticulum (ER-) stress. In our study, estrogen and progesterone reduced ER-stress and pro-inflammatory cytokine production while wound healing and barrier function of epithelial cells was increased. An inflammatory phenotype in our study was mimicked by treatment of cells with tunicamycin, which induces ER stress. We felt the need to do so as in our study comparison of organoids from inflamed and non-inflamed intestinal tissue biopsies demonstrated no differences in barrier strength through upregulation of tight junctions or IL-8 cytokine production. Since our study, a new study underlined the importance of re-inducing inflammation in organoids derived from biopsies, as they lose their transcriptional inflammatory phenotype over time(16). Future studies might assess the effect of inflammation modelled in organoid models based on treatment with inflammatory microbiome or cytokines.
Overall we suggested with this study that pregnancy hormones can have a direct positive effect on disease activity. Estrogen receptor (ER)-beta signaling is known to be reduced in patients with IBD, although a recent study shows differences in the expression of receptors depending on the sex and age of patients (17). In addition to the nuclear estrogen receptors, membrane-bound estrogen receptors and their effect on IBD was recently studied by

Jacenik et al. (18). They demonstrate a mediation of the G-protein coupled estrogen receptor (GPER) in anti-inflammatory actions of estrogen. In an IBD mouse model they show that activation of GPER reduced mortality, improved macro- and microscopic scores and lowered C-reactive protein levels. Interestingly, the membrane-bound GPER are enhanced in CD, but not UC patients, suggesting rising estrogen levels during pregnancy may potentially have a higher beneficial effect in CD patients as compared to UC patients. Thus, in addition to changes in Th-cytokine patterns, CD patients may derive more benefit from pregnancy-induced changes via direct stimulation of mucosal estrogen receptors.

In CHAPTER 4, we studied immunological and microbial changes that take place during pregnancy. In addition to immunological changes, the intestinal microbiome is also altered during healthy pregnancy. Studies describe a reduction of the microbial diversity in the third trimester and third trimester stool microbiota showing inflammatory characteristics (19,20). In non-pregnant IBD patients, alterations in the microbiome are also present, most importantly a reduced fecal bacterial diversity (21–23). In our study we describe more subtle changes in bacterial features of the microbiome of IBD patients as pregnancy progressed. In other words, IBD-related dysbiosis disappears during middle and late pregnancy to a diversity level seen in healthy pregnancy. In addition we found that the IBD microbiomes were more similar to one another, which was unexpected.

It is possible to differentiate between CD and UC based on microbiome signature (24). In our study we found a reduction of microbial differences between UC and CD during pregnancy. Differences between CD patients experiencing a flare during pregnancy and those who did not were larger than flaring and non-flaring UC patients, suggesting that microbiome in CD patients is more susceptible to modulation. However it should be noted that more CD patients were included in the study and that perhaps the effects for UC patients were underestimated.

PART II BEDSIDE

The first four chapters described the changes taking place during pregnancy and the effect on hormonal, microbial and immunological level. Overall, the data obtained in our laboratory studies suggest that immunological, epithelial barrier and microbial changes may take place during pregnancy, which all point toward a beneficial effect of pregnancy on the course of IBD. In particular CD patients are likely to benefit on the basis of these findings. However, to what extent these molecular alterations contribute to changes in disease course remained unclear. The aim of this thesis is also to describe the clinical course and investigate if the positive changes described in the first part of this thesis translate to an altered disease course, as the effect of pregnancy on the clinical course of IBD post-partum

and whether the hormonal, microbial and immunological changes have a long lasting effect thus far remains controversial. In CHAPTER 5 we studied the effect of pregnancy on the risk of relapse in primigravida IBD patients and described the disease course before and after a first pregnancy. We found lower relapse rates per person per year in the 4 years after delivery compared to the 3 years before a first pregnancy. Especially for CD patients, pregnancy seems to have a positive effect on the course of IBD. In contrast, UC patients were more at risk of relapsing post-partum and compared to CD patients UC patients relapsed also more during pregnancy.

The risk of relapse during pregnancy is increased by disease activity prior to pregnancy and therefore is it advised to strive a remission of at least 6 months prior to conception (25,26). Again, this is underlined in a recent study where it was demonstrated that not only disease activity at the time of conception was an predictor of disease relapse during pregnancy, but an increased risk of relapse in the second pregnancy was associated with a history of a relapse in the prenatal course of prior pregnancy (27).

The importance of good counseling is proven by thorough research (28). In order to give good and complete counseling, the potential effect of IBD on children born to IBD mothers is of equal importance. Most studies thus far have focused on the clinical health outcomes of children born to these mothers. These outcomes are reassuring (29). However, IBD medication and treatment of patients is subject to change, and in order to achieve remission new medication is being used (30). The effect of these medicines on pregnancy outcome and the developing immune system of the children remains uncertain and therefore topic for future research.
On the psychological health of the children born to IBD mothers there is also limited data. A study from 2013 described no difference in psychological health of 30 children exposed to intrauterine thiopurines compared to a historical control group (31). In CHAPTER 6 of this thesis we aimed to extend this knowledge and studied the health-related-quality of life (HRQoL) of children (1-5 years) born to mothers with IBD. Reassuringly we showed that the HRQoL of these children, as reported by the parents, is comparable to the quality of life of children born to mothers without IBD. In addition, IBD related factors (e.g. IBD subtype, IBD surgery prior to pregnancy and disease activity during pregnancy) did not influence the HRQoL of the children. Follow-up of these children until adulthood is warranted, as studies show impact on the quality of life in this age-group. Young adults (aged 16-25 years) indicate that they experience negative consequences in their daily life when growing up with a chronically ill family member (32). Furthermore, a study published in 2017 on the effect of parental chronical illness on emerging adults, with a mean age of 19.5 years, described a significantly higher measurement of quality of life in participants without chronically ill parents. In addition, participants with chronically ill parents scored significantly lower on multiple domains of psychosocial functioning (e.g. elevated depression, anxiety, stress and lack of optimism) (33).

Overall, we concluded in this thesis that the immune regulation in IBD and pregnancy is complicated and that the effect of pregnancy on the course of IBD is dependent on individual patient's characteristics (i.e. the course of IBD before pregnancy, microbiome and hormonal changes).

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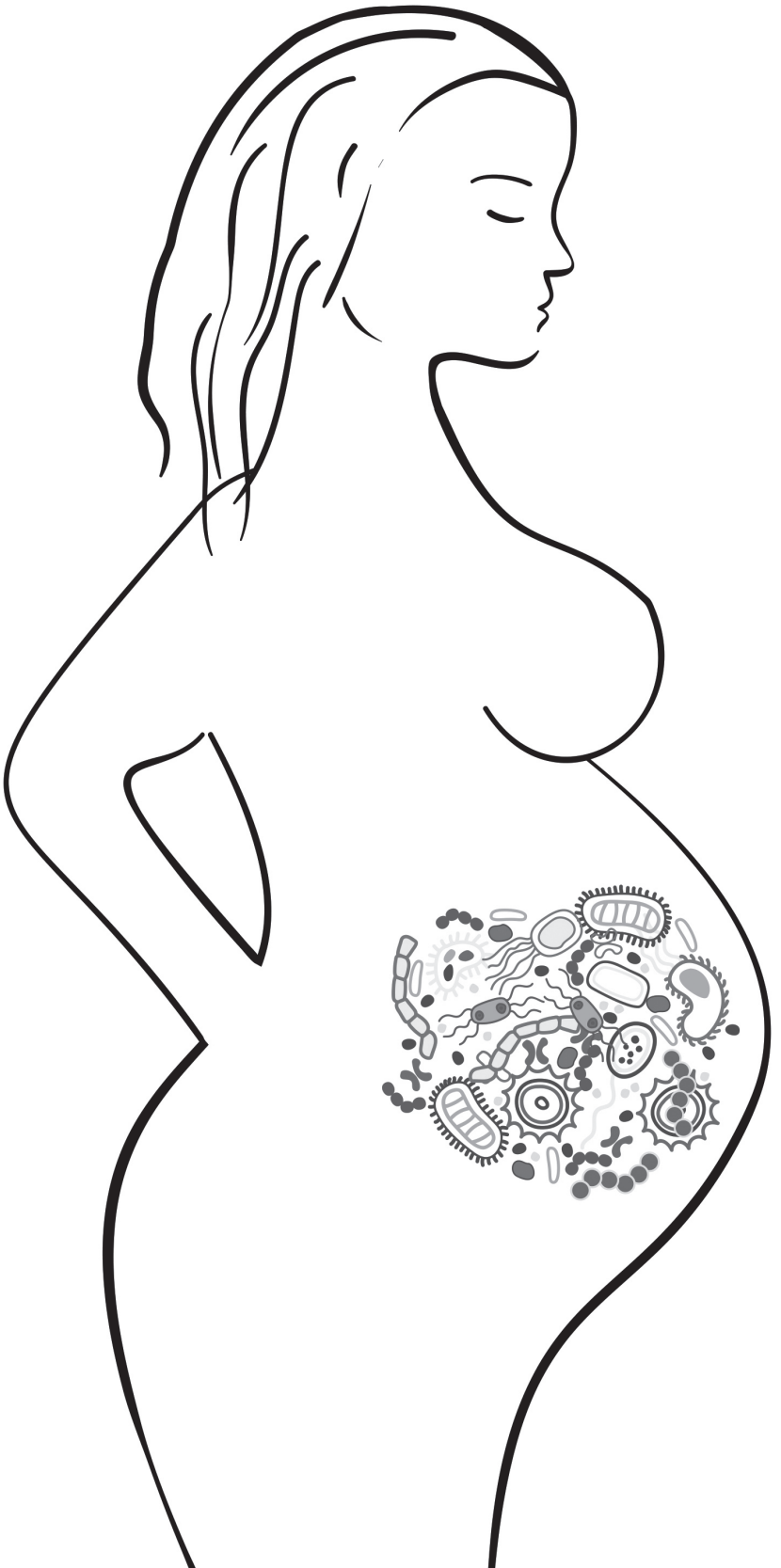


CHAPTER 8

Nederlandse samenvatting

Het doel van dit proefschrift is het uitbreiden van fundamentele en klinische kennis over Inflammatoire darmziekten (IBD) en zwangerschap.
IBD wordt vaak gediagnosticeerd op jonge, tevens vruchtbare, leeftijd. Er ontstaan daarom vragen en er is bezorgdheid over hoe IBD zwangerschap beïnvloed en hoe zwangerschap het beloop van IBD beïnvloed. Er is een verband tussen matige tot ernstige IBD en lagere geboortecijfers, toename van spontane abortus en keizersnede en daarom is deze bezorgdheid gegrond. Het is dan ook van belang onze kennis over zwangerschap-gerelateerde fysiologie van IBD patiënten uit te breiden en te streven naar een gezonde zwangerschap. Door een beter inzicht te krijgen in de fysiologie en pathologie van zowel zwangerschap als IBD, kunnen we mogelijk in de toekomst voorspellen welke patiënten een verminderde of verhoogde ziekteactiviteit zullen ervaren tijdens en na de zwangerschap.
In het eerste deel van dit proefschrift hebben wij de moleculaire basis, de microbiële en hormonale veranderingen, het effect op de epitheliale barrière en het immuun systeem beschreven, die plaatsvinden tijdens zwangerschap in IBD patiënten. In het tweede deel van dit proefschrift beschrijven we klinische effecten van de veranderingen die beschreven worden in het eerste deel, waarbij ook rekening wordt gehouden met het mogelijke effect op de kwaliteit van leven van kinderen van deze moeders.

DEEL 1 FUNDAMENTEEL ONDERZOEK

Tijdens een zwangerschap vinden er hormoon-geïnduceerde veranderingen en aanpassingen van het immuunsysteem plaats. In HOOFDSTUK 2 bespreken we deze veranderingen en welk mogelijke effect dit heeft op het beloop van IBD. Tijdens de zwangerschap kan de 'lichaamsvreemde' foetus groeien omdat het immuunsysteem van de moeder verminderde herkenning en reactie op foetale cellen heeft. Er is ook een verschuiving van het cytokinepatroon tijdens de zwangerschap, met een pro-inflammatoir Th1-cytokinepatroon tijdens de implantatie, gevolgd door een verschuiving naar het Th2-fenotype tijdens het grootste gedeelte van de zwangerschap en opnieuw een Th1-fenotype tijdens de bevalling. Er wordt gespeculeerd dat deze brede verschuiving naar een Th2-fenotype gedurende het grootste deel van de zwangerschapsperiode in het voordeel is van Th1-gemedieerde ziekten zoals CD. Het is nog onduidelijk in hoeverre deze lokale veranderingen effect hebben op de rest van het lichaam.
In HOOFDSTUK 4 beschrijven we dat cytokine levels in het perifere bloed bij IBD-patiënten significant en ten gunste veranderen van vóór tot tijdens de zwangerschap, wat suggereert dat een zwangerschap inderdaad het ziektebeloop van IBD positief kan beïnvloeden. Wat betreft immunologische veranderingen lieten we een afname zien van pro-inflammatoire cytokines (IL-6, IL-8, IL-12, IL-17 en TNF-alfa) rondom conceptie, terwijl tijdens de zwangerschap de cytokinespiegels stabiel bleven. We hebben echter geen specifieke Th1- of Th2-verschuiving waargenomen, noch hebben we in deze studie onderscheid kunnen maken tussen CD en UC patiënten op basis van het cytokineprofiel.

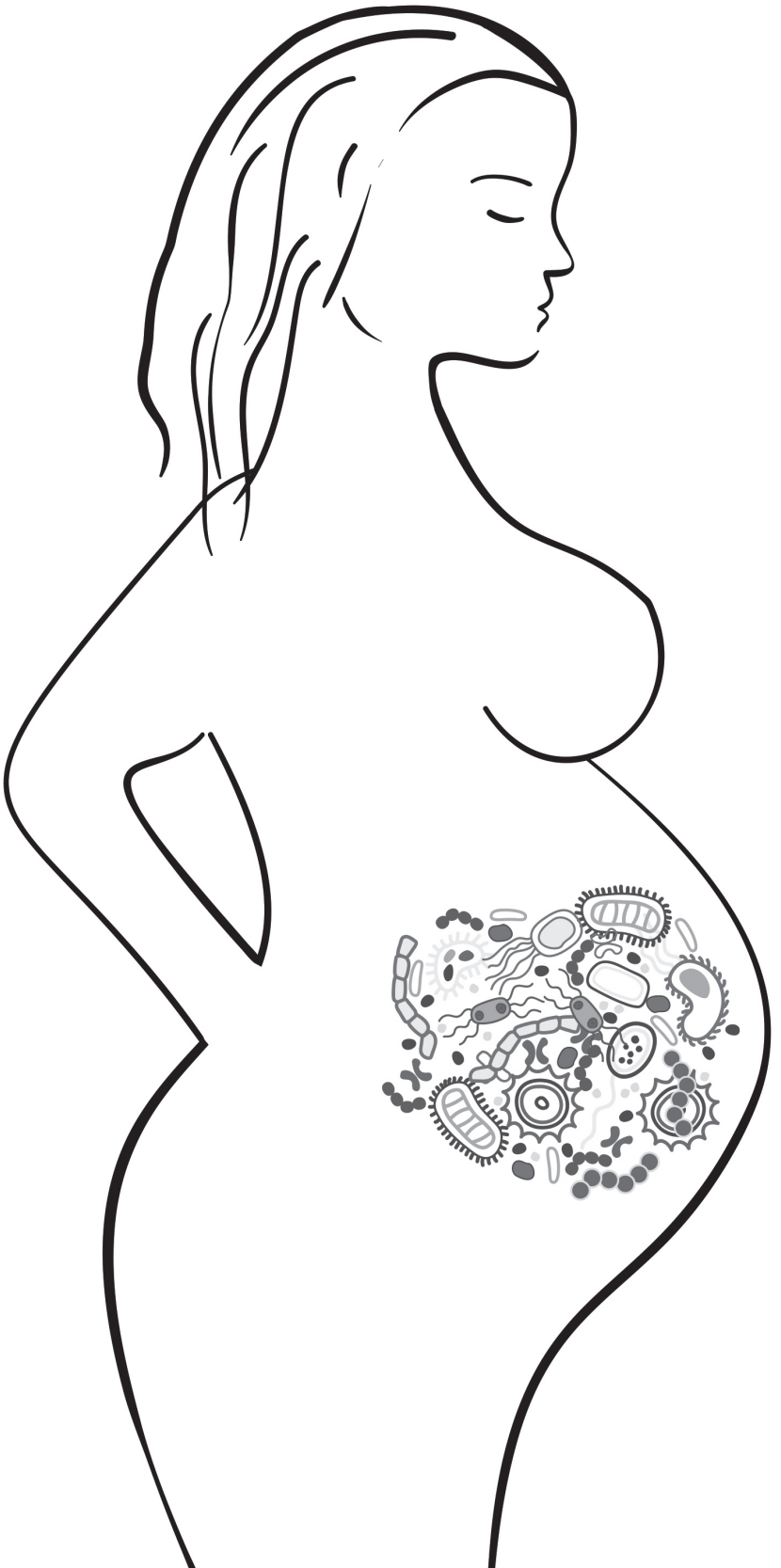
Gedurende de zwangerschap nemen de hormonen oestrogeen en progesteron toe, tot in het derde trimester. Studies suggereren anti-inflammatoire effecten van deze hormonen door het verminderen van inflammatoire cytokines. Zowel klinische studies als dierstudies naar het effect van deze hormonen op het beloop van IBD zijn echter tegenstrijdig. Bovendien zijn de dierstudies moeilijk te vertalen naar de zwangere IBD-patiënten en bleef hierin het directe effect van de hormonen onduidelijk. Daarom onderzochten wij in HOOFDSTUK 3 het directe effect van oestrogeen en progesteron op darmepitheel cellen en hun functie. De epitheliale barrière is de eerste verdedigingslinie tegen binnendringende micro-organismen. Bij IBD-patiënten produceren immuuncellen inflammatoire cytokines, zoals IFN-gamma en TNF-alfa, waardoor een proces ontstaat dat de 'leaky gut' wordt genoemd. Deze verzwakte darmbarrièrefunctie is een belangrijk aspect van de pathofysiologie van IBD. Tijdens actieve ziekte nemen ook de zogenaamde 'tight junctions' af, deze van invloed zijn op de regulering van epitheliale permeabiliteit. Bovendien ontstaat er zogenaamde endoplasmatische reticulum (ER-) stress, hierbij ontstaat er een opeenhoping van ongevouwen eiwitten in het endoplasmatisch reticulum. Dit proces is het gevolg van inflammatoire triggers die het juist vouwen van deze eiwitten bij IBD belemmeren. In onze studie verminderden oestrogeen en progesteron ER-stress en pro-inflammatoire cytokineproductie, terwijl wondgenezing en barrièrefunctie van epitheelcellen verbeterden. Samenvattend, suggereren wij met deze studie dat zwangerschapshormonen een positief effect kunnen hebben op de ziekteactiviteit.

In HOOFDSTUK 4 hebben we immunologische en microbiële veranderingen bestudeerd die plaatsvinden tijdens de zwangerschap. Naast immunologische veranderingen verandert ook het darm-microbioom tijdens een gezonde zwangerschap. Tijdens het derde trimester is er een vermindering van de microbiële diversiteit en zijn er inflammatoire kenmerken te herkennen in de ontlasting. In onze studie beschrijven we meer subtiele veranderingen in bacteriële kenmerken van het microbioom van IBD-patiënten naarmate de zwangerschap vorderde. Met andere woorden, IBD-gerelateerde dysbiose verdwijnt tijdens midden en late zwangerschap tot een diversiteitsniveau dat wordt gezien bij een gezonde zwangerschap. Daarnaast vonden we ook een lagere bèta-diversiteit, wat betekent dat de IBD-microbiomen meer op elkaar leken.
Het is ook mogelijk om onderscheid te maken tussen CD en UC op basis van het microbioom. In onze studie vonden we een vermindering van microbiële verschillen tussen UC en CD tijdens de zwangerschap. Verschillen tussen CD-patiënten met en CD-patiënten zonder opvlamming waren groter dan de verschillen tussen deze twee groepen bij UC-patiënten, wat suggereert dat het microbioom van CD-patiënten gevoeliger is voor veranderingen. Er moet echter worden opgemerkt dat er meer CD-patiënten in de studie waren opgenomen en dat de effecten voor UC-patiënten daardoor mogelijk werden onderschat.

DEEL 2 KLINISCH ONDERZOEK

De eerste vier hoofdstukken beschrijven de veranderingen die plaatsvinden tijdens de zwangerschap en het effect op hormonaal, microbieel en immunologisch niveau. Concluderend suggereren wij met de data uit onze studies dat immunologische veranderingen, veranderingen van de epitheliale barrière en microbioom tijdens de zwangerschap een positief effect kunnen hebben op het beloop van IBD. Op basis van deze bevindingen, lijken met name CD patiënten hiervan de positieve effecten te kunnen ondervinden. In hoeverre deze moleculaire veranderingen bijdragen aan veranderingen in het ziektebeloop is niet duidelijk. Een tweede doel van dit proefschrift is daarom het klinische beloop te beschrijven en te onderzoeken of de positieve veranderingen beschreven in het eerste deel van dit proefschrift zich vertalen in een veranderd ziektebeloop.
In HOOFDSTUK 5 hebben we het effect van zwangerschap op het risico van een opvlamming bij primigravida IBD patiënten bestudeerd en het ziektebeloop voor en na een eerste zwangerschap beschreven. We vonden een lager aantal opvlammingen per persoon per jaar in de 4 jaar na de bevalling vergeleken met de 3 jaar vóór een eerste zwangerschap. Vooral bij CD patiënten lijkt zwangerschap een positief effect te hebben op het beloop van IBD. Daarentegen liepen UC patiënten meer risico om postpartum een opvlamming te krijgen en vergeleken met CD patiënten kregen meer UC patiënten tijdens de zwangerschap een opvlamming.
Goede begeleiding vóór, tijdens en na de zwangerschap ik van groot belang. Om een goede en volledige begeleiding te geven, is het potentiële effect van IBD op kinderen van IBD-moeders even belangrijk. Tot dusver hebben de meeste onderzoeken zich gericht op de klinische gezondheidsresultaten van kinderen van deze moeders, terwijl over de psychische gezondheid van de kinderen van IBD-moeders beperkte gegevens zijn. In HOOFDSTUK 6 van dit proefschrift hebben we geprobeerd deze kennis uit te breiden en de gezondheid gerelateerde kwaliteit van leven (HRQoL) van kinderen (1-5 jaar) van een moeder met IBD onderzocht. Wij lieten zien dat de HRQoL van deze kinderen, zoals gerapporteerd door de ouders, vergelijkbaar is met de kwaliteit van leven van kinderen van moeders zonder IBD. Bovendien hadden IBD-gerelateerde factoren (IBD subtype, IBD operatie voorafgaand aan de zwangerschap en ziekteactiviteit tijdens de zwangerschap) geen invloed op de HRQoL van de kinderen. Follow-up van deze kinderen tot de volwassen leeftijd is aan te bevelen, aangezien onderzoeken een impact laten zien op de kwaliteit van leven in deze leeftijdsgroep.

Samenvattend concluderen we in dit proefschrift dat de immuun regulatie bij IBD en zwangerschap gecompliceerd is en dat het effect van zwangerschap op het beloop van IBD afhankelijk is van de individuele kenmerken van de patiënt (d.w.z. het beloop van IBD vóór de zwangerschap, microbioom en hormonale veranderingen).



Appendices

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List of publications

PhD portfolio

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Curriculum Vitae

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List of publications

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Journal of Translational Medicine 2018 Mar;16(1):55
[1]Contributed equally

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Fecal Matrix Metalloproteinase-9 measurement for optimizing detection of disease activity in Inflammatory Bowel Disease.
Journal of Clinical Gastroenterology 2019 May;53(5):395-397
[1]Contributed equally

PhD portfolio

Courses

2015 Good Clinical Practice (BROK), Examenbureau Medisch Wetenschappelijk Onderzoeker, Medical Center Alkmaar, Alkmaar, The Netherlands

2016 Basic Introduction on SPSS, Molecular medicine postgraduate school, Erasmus University Medical Center, Rotterdam, The Netherlands

2016 Systematic Literature Retrieval in PubMed and other databases, Erasmus University Medical Center, Rotterdam, The Netherlands

2016 Introduction in GraphPad Prism, Molecular medicine postgraduate school, Erasmus University Medical Center, Rotterdam, The Netherlands

2017 Integrity in scientific research, Dept. of Medical ethics and Philosophy, Erasmus University Medical Center, Rotterdam, The Netherlands

2018 Biomedical English Writing Course, Molecular medicine postgraduate school, Erasmus University Medical Center, Rotterdam, The Netherlands

Oral presentations

2017 Pregnancy in IBD: direct effect of sex-hormones on epithelial barrier function. Digestive Disease Days, The Dutch Association for Gastroenterology and Hepatology, Veldhoven, The Netherlands

2017 Thiopurine dose adjustment during pregnancy in inflammatory bowel disease: a case series. The Dutch Association for Gastroenterology and Hepatology, Veldhoven, The Netherlands

2017 Care for pregnant IBD patients. IBD day, Leiden, The Netherlands

2017 Post-pregnancy era. IBD Delta Day, Rotterdam, The Netherlands

2018 A first pregnancy seems associated with a positive effect on the course of inflammatory bowel disease: data from a prospective pregnancy cohort. Digestive Disease Days, The Dutch Association for Gastroenterology and Hepatology, Veldhoven, The Netherlands

Poster presentations

2017 Pregnancy in IBD: direct effect of sex-hormones on epithelial barrier function. European Crohn's and Colitis Organisation (ECCO), Barcelona, Spain

2018 A first pregnancy seems associated with a positive effect on the course of inflammatory bowel disease: data from a prospective pregnancy cohort. European Crohn's and Colitis Organisation (ECCO), Vienna, Austria

2019 Modulation of cytokine patterns and microbiome during pregnancy in Inflammatory bowel disease. European Crohn's and Colitis Organisation (ECCO), Copenhagen, Denmark

Attended (inter)national conferences

2016 11th congress of European Crohn's and Colitis Organisation (ECCO), Amsterdam, The Netherlands
- Y-ECCO workshop (review an article)
- Post-ECCO meeting, Amsterdam, The Netherlands

2016 Women and Their Microbes congres, Amsterdam, The Netherlands

2017 12th congress of European Crohn's and Colitis Organisation (ECCO), Barcelona, Spain
- Y-ECCO workshop (grand proposal)
- Post-ECCO meeting, Amsterdam, The Netherlands

2017 Half-yearly Meeting of the Dutch Association of Gastroenterology, Veldhoven, The Netherlands

2017 ICC day, Amsterdam, The Netherlands

2018 13th congress of European Crohn's and Colitis Organisation (ECCO), Vienna, Austria

2018 Half-yearly Meeting of the Dutch Association of Gastroenterology, Veldhoven, The Netherlands

2019 14th congress of European Crohn's and Colitis Organisation (ECCO), Copenhagen, Denmark

2019 ICC day, Amsterdam, The Netherlands

Reviewing for scientific journals

Gastroenterology, Journal of Crohn's and Colitis (JCC) and Gut.

Teaching

2017 Lecture on IBD and pregnancy at the Midwifery Academy, Rotterdam

2017 Supervision of a student Biology and Medical Laboratory research, Hogeschool Rotterdam

2017 Supervision of two students Microbiology, Technical College Rottedam

2018 Supervision scientific research project of a master student Medical School, Erasmus Medical Center, Rotterdam

2018 Supervision of a master student Biology and Medical Laboratory research, Hogeschool Rotterdam

Memberships

Dutch Society of Gastroenterology (NVGE)

European Crohn's and Colitis Organisation (ECCO)

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Alles komt goed! Zo niet – dan toch!

Curriculum Vitae

Jannie (Janine) van der Giessen was born on December 27th 1988 in Den Helder, The Netherlands. After graduating high school in 2007, she studied Biomedical Sciences at the Vrije Universiteit Amsterdam and completed the first year. In 2008 she started studying Medicine at the Vrije Universiteit Amsterdam. She completed her master thesis at the department Gastroenterology and Hepatology of the Medical Center Alkmaar and worked under supervision of dr. M. Klempt-Kropp. In December 2014 she obtained her medical degree and started working in the Medical Center Alkmaar at the department of Internal Medicine and Gastroenterology and Hepatology as a medical doctor for over a year. In March 2016 she started her PhD at the Erasmus Medical Center Rotterdam, under supervision of prof. C.J. van der Woude, prof. M.P. Peppelenbosch and dr. G.M. Fuhler, which resulted in this thesis. In July 2019 she started her residency in Gastroenterology and Hepatology at the department of Internal Medicine at the Erasmus Medical Center Rotterdam and will continue her residency in this hospital at the department Gastroenterology and Hepatology from January 2021 and onwards. Together with Ruben, Janine lives in Middenbeemster.