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Celiac disease and reproductive disorders: meta-analysis of epidemiologic associations and potential pathogenic mechanisms

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BACKGROUND: An increased risk of reproductive failures in women with celiac disease (CD) has been shown by several studies but a comprehensive evaluation of this risk is lacking. Furthermore, the pathogenic mechanisms responsible for obstetric complications occurring in CD have not been unraveled.

METHODS: To better define the risk of CD in patients with reproductive disorders as well as the risk in known CD patients of developing obstetric complications, we performed an extensive literature search of Medline and Embase databases. Odds ratio (OR) and relative risk (RR) with 95% confidence intervals (95% CI) were used in order to combine data from case–control and cohort studies, respectively. All data were analyzed using Review Manager software. In addition, we summarized and discussed the current hypotheses of pathogenic mechanisms potentially responsible for obstetric complications occurring in CD.

RESULTS: Patients with unexplained infertility, recurrent miscarriage or intrauterine growth restriction (IUGR) were found to have a significantly higher risk of CD than the general population. The OR for CD was 5.06 (95% CI 2.13–11.35) in patients with unexplained infertility, 5.82 (95% CI 2.30–14.74) in women experiencing recurrent miscarriage and 8.73 (95% CI 3.23–23.58) in patients with IUGR. We did not observe an increased risk of CD in women delivering small-for-gestational age or preterm babies. Furthermore, we found that in celiac patients, the risk of miscarriage, IUGR, low birthweight (LBW) and preterm delivery is significantly higher with an RR of 1.39 (95% CI 1.15–1.67), 1.54 (95% CI 1.22–1.95), 1.75 (95% CI 1.23–2.49) and 1.37 (95% CI 1.19–1.57), respectively. In addition, we observed that the risk for IUGR, LBW and preterm delivery was significantly higher in untreated patients than in treated patients. No increased risk of recurrent miscarriage, unexplained

stillbirth or pre-eclampsia was found in celiac patients. *In vitro* studies have provided two main pathogenic models of placental damage at the feto-maternal interface. On the embryonic side of the placenta, a direct binding of anti-transglutaminase (-TG) antibodies to trophoblast cells and, thus, invasiveness reduction via an apoptotic damage, has been proposed. Anti-TG antibodies may also be detrimental to endometrial angiogenesis as shown *in vitro* in human endometrial endothelial cells (cultures and *in vivo* in a murine model). The angiogenesis inhibition seems to be the final effect of anti-TG antibody-mediated cytoskeletal damage in endometrial endothelial cells.

CONCLUSIONS: Physicians should investigate women with unexplained infertility, recurrent miscarriage or IUGR for undiagnosed CD. Women with CD show an increased risk of miscarriage, IUGR, LBW and preterm delivery. However, the risk is significantly reduced by a gluten-free diet. These patients should therefore be made aware of the potential negative effects of active CD also in terms of reproductive performances, and of the importance of a strict diet to ameliorate their health condition and reproductive health. Different mechanisms seem to be involved in determining placental tissue damage in CD patients.

Key words: celiac disease / pregnancy / trophoblast / endometrium / angiogenesis

Introduction

Celiac disease (CD) is an autoimmune enteropathy caused by an abnormal immune response to dietary gluten, the protein fraction of wheat, barley and rye, in genetically susceptible individuals. The genetic susceptibility to develop CD has been shown to be conferred by HLA class II molecules DQ2 or DQ8, responsible for presenting disease-related peptides to T lymphocytes. The exposure of the immune system to the immunogenic and toxic peptides of gliadin, the alcohol-soluble fraction of gluten, can promote an inflammatory reaction. Undigested molecules of gliadinin in conditions of increased intestinal permeability pass through the epithelial barrier of the intestine and interact with antigen-presenting cells (APCs) in the lamina propria (Sollid, 2002). The adaptive immune response is mediated by $CD4^+$ T lymphocytes in the lamina propria that recognize gliadin peptides, bound to HLA-DQ2 and -DQ8 molecules on APCs, leading to production of the pro-inflammatory cytokine interferon-γ (Salvati *et al.*, 2005) and to a B lymphocyte response that results in production of autoantibodies like endomysial, anti-transglutaminase (TG) and anti-gliadin antibodies (Jabri and Sollid, 2006).

CD occurs in adults and children at rates approaching 1% of the general population but only 20–50% of affected individuals have subjective symptoms (Fasano *et al.*, 2003; Mäki *et al.*, 2003; West *et al.*, 2003; Bingley *et al.*, 2004; Tatar *et al.*, 2004). The symptoms of the classical form of childhood CD are malabsorption related and include chronic diarrhea, steatorrhea, abdominal distension, fatigue, nausea, vomiting, anemia and growth retardation. However, CD can present with several non-gastrointestinal symptoms and it may escape timely recognition (Eliakim and Sherer, 2001; Murray *et al.*, 2003; Mäki *et al.*, 2004). Thus, given the heterogeneity of clinical presentation, many atypical cases of CD go undiagnosed, leading to a risk of long-term complications. Among atypical symptoms of CD, disorders of fertility, such as delayed menarche, early menopause, amenorrhea or infertilit, and pregnancy complications, such as recurrent abortions, intrauterine growth restriction (IUGR), small for gestational age (SGA) babies, low birthweight (LBW) babies or preterm deliveries, must be factored (Eliakim and Sherer, 2001) (see more below).

Endomysial and anti-TG antibodies are considered the most sensitive serologic test to screen for CD and the sensitivity of the tests for both autoantibodies is greater than 90% (Rostom *et al.*, 2005). Currently, a test for either marker is considered to be the best means of screening for CD and to identify the individuals to be referred for endoscopy (Rostom *et al.*, 2005). The gold-standard treatment for CD relies on a lifelong gluten-free diet (GFD), which interrupts the immune response triggered by gluten (Tack *et al.*, 2010).

A closer examination of pathogenic mechanisms and clinical management of CD goes beyond the scope of this discussion and the reader is invited to refer to the increasing number of excellent reviews on the field (Ciccocioppo *et al.*, 2005; Sollid and Jabri, 2005; Green and Cellier, 2007).

In this review, we focus on the impact of CD on the reproductive health of women, providing a comprehensive review and meta-analysis of the literature investigating the effect of CD on pregnancy outcomes as well as on the incidence of undiagnosed CD in cohorts of women with a history of obstetric failures. Furthermore, we extensively discuss current hypotheses of the pathogenic mechanisms involved in the occurrence of placental-related complications in women with CD.

CD and reproductive disorders: systematic review and meta-analysis

During the last decades, the association between CD and a wide range of reproductive disorders has been described in a growing number of papers and it is now well recognized that CD may have implications on women's reproductive health.

A shorter duration of the fertile life span in women with untreated CD, because of an older age of menarche and a younger age of menopause, and an increased prevalence of secondary amenorrhea, have been shown in several studies (Ferguson *et al.*, 1982; Sher and Mayberry, 1996; Smecuol *et al.*, 1996; Santonicola *et al.*, 2011) and only one study failed to confirm this observation (Sferlazzas *et al.*, 2008). Interestingly, the contraction of the reproductive period seems to be directly related to the activity of CD, since before-and-after case–control studies have shown that celiac women on a long-term GFD show a duration of fertile life span analogue to healthy women (Ferguson *et al.*, 1982; Smecuol *et al.*, 1996; Santonicola *et al.*, 2011).

To date, it is also widely accepted that untreated CD also represents a risk for a short-breastfeeding period. In a case–control before–after study, Ciacci *et al.* demonstrated that the duration of breastfeeding was 2.5 times shorter in untreated celiac mothers than in healthy

women. Again, a central role for gluten exposition and CD activity has been proposed for this reproductive disorder, since the authors also demonstrated that the introduction of a GFD increased the duration of breastfeeding by 2.4 times, restoring it to the average value of the general female population (Ciacci *et al.*, 1996).

Several authors have also investigated the fertility rate of women affected by CD. In particular, in a large Swedish population-based cohort study assessing fertility in women with CD compared with controls, the overall fertility rate of the two groups was similar but the fertility of celiac women was decreased in the 2 years preceding CD diagnosis (Zugna *et al.*, 2010).

Consistent with this, Sher and Mayberry observed that the mean number of children born to celiac patients was significantly less when compared with controls before diagnosis while, after diagnosis and treatment, patients had a number of children similar to controls. The authors concluded that the overall difference in fertility between celiac women and controls was due to relative infertility prior to diagnosis and its correction by a GFD (Sher and Mayberry, 1996). These data strongly indicate that reduced fertility is more common in patients with active CD when a GFD is unlikely to have been initiated.

In addition to the reproductive disorders considered above, most of the research has focused on the association between CD and adverse pregnancy outcomes. Indeed, celiac women have been found to have a higher risk of pregnancy complications in their reproductive life and several studies have shown that CD is detectable at an increased frequency in some high-risk groups of patients with a history of reproductive failures.

Among all of the reproductive disorders referable to CD, unexplained infertility, recurrent pregnancy loss, stillbirth, IUGR and LBW of babies have been most investigated.

Since the evidence of these associations has come mainly from case–control and cohort studies and results arising from these studies are still controversial, we performed a meta-analysis of the studies published in international journals to clarify the relationship between CD and increased risk of reproductive failures. We investigated both the prevalence of CD in women with reproductive disorders, as well as, from another point of view, the incidence of obstetric complications in women with CD compared with controls.

Methods

We performed an extensive literature search of Medline and Embase Current Contents databases from inception until December 2012, using a broad combination of search terms for CD (e.g. CD, anti-TG antibodies, gluten, gliadin) and selected adverse reproductive outcomes (e.g. unexplained infertility, miscarriage, recurrent miscarriage, unexplained stillbirth, IUGR, LBW, SGA) that are more frequently associated with CD and for which enough data were available to make a significant comparison between different studies. We also reviewed the reference lists of all identified studies and review articles to search for additional references.

The eligibility of the studies was independently assessed by two researchers (C.T. and C.W.) in order to select case–control studies reporting the occurrence of CD in women with and without reproductive disorders and cohort studies yielding the incidence of reproductive disorders in women with and without CD. Duplicates of studies were removed. In the first step, the studies were excluded considering only the information presented in the title and abstract. In the second step, the full texts of the articles not previously excluded were assessed to determine their eligibility for the review, using the same set of criteria. In the third step, the full texts were re-evaluated to determine the eligibility for meta-analyses (Fig. 1).

To assess the comparability of the selected studies, we verified that the same definitions of the outcomes analyzed were used.

Unexplained infertility defines the absence of pregnancy after at least 2 years of regular sexual intercourse with: normal semen analysis from the husband (World Health Organization criteria, 1999); normal ovulation assessed by progesterone values and/or premenstrual endometrial biopsy; normal post-coital test results (for cervical factor of infertility); normal serum LH, FSH and PRL levels; normal tubal patency assessed by hysterosalpingography/laparoscopic chromotubation and normal results of diagnostic laparoscopy.

Miscarriage is defined as a spontaneous pregnancy loss occurred within the first 24 weeks of gestation. Recurrent miscarriage refers to the occurrence of two or more spontaneous pregnancy losses. Unexplained stillbirth is the birth of a new born after 24 completed weeks of gestation that did not show any signs of life after delivery in the absence of a recognizable cause. IUGR defines, according to the national growth curves of the Countries where the studies were conducted, a fetal weight for age <2 standard deviations (SD) from the mean. Infants with a birthweight below the 10th percentile weight for gestational age with respect to the reference birthweight values are defined as SGA. LBW is used to define a birthweight <2500 g at term.

We excluded non-English language papers. We did not include conference abstracts where more detailed papers describing the same study were unavailable. Studies designed without a control group were not included in the meta-analysis. Studies were classed as higher quality and worthy of selection if they satisfied all of the selection criteria. There were 15 case–control and 9 cohort studies included in the meta-analysis. Because of the small number of studies available, a quality assessment of the single studies was not performed. The risk of publication bias was evaluated by funnel plots.

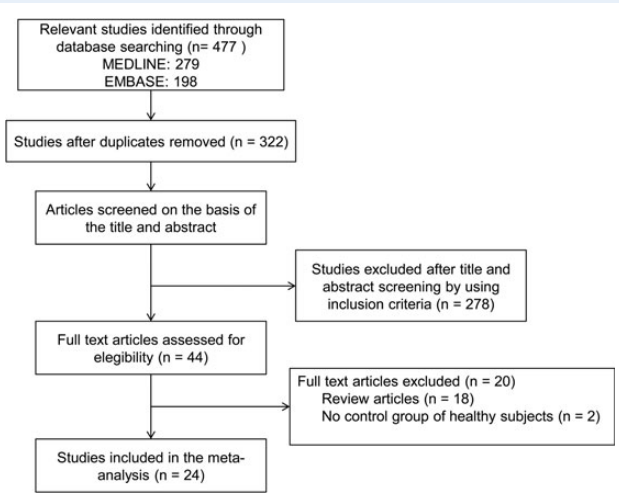

Figure 1 Flow chart showing the search strategy and steps of study selection for the meta-analysis.

Data extraction from selected papers was performed independently by two researchers as follows. For case–control studies papers were first divided into groups depending on the reproductive disorder evaluated (seven groups: IUGR, recurrent miscarriage, SGA, unexplained stillbirth, unexplained infertility, pre-eclampsia, preterm delivery), then data were extracted as the number of celiac cases in the total number of women with a specific reproductive disorder and as the number of celiac cases in the total number of control women. For cohort studies the papers were first divided according to the considered reproductive disorder (eight groups: SGA, IUGR, recurrent miscarriage, miscarriage, unexplained stillbirth, LBW, preterm delivery, pre-eclampsia) and data were extracted as the number of cases of a specific reproductive disorder among celiac women in the total number of celiac women and as the number of cases of the disorder among control women in the total number of control women. Odds ratio (OR) or relative risk (RR) with 95% confidence intervals (95% CI) was used in order to analyze the data from case–control and cohort studies, respectively. Several forest plots were obtained with respect to the different end-points considered in this review.

In the combination of case–control studies, the OR of having CD was calculated for each of the end-points, whereas, in the analysis of cohort studies, RRs were calculated for each single end-point for celiac patients in comparison with controls.

The analysis of cohort studies was also stratified according to the current treatment of patients; for these analyses only studies reporting data for both treated and untreated patients were used.

The Mantel–Haenszel fixed-effects model was applied to combine data if heterogeneity was not shown. On the contrary, the DerSimonian and Laird random-effects model was used. The Q test and the I^2 statistics were used to assess heterogeneity: a P-value < 0.10 or an $I^2 > 50\%$ was considered indicative of substantial heterogeneity. Analyses were performed using Review Manager software.

Results

The combination of data from case–control studies was performed with respect to all cases considered together in comparison with controls (Fig. 2A) and for patients with unexplained infertility (Fig. 2B), recurrent miscarriage (Fig. 2C), IUGR (Fig. 3A), SGA babies (Fig. 3B) or preterm delivery (Fig. 3C). Overall, the OR for CD in all cases was 4.97 (95% CI 2.88–8.57). All the considered reproductive failures, except cases of SGA and preterm delivery, were shown to be at a significant higher risk of CD. The OR for CD was 5.06 (95% CI 2.13–11.35) in patients with unexplained infertility, 5.82 (95% CI 2.30–14.74) for women experiencing recurrent miscarriage and 8.73 (95% CI 3.23–23.58) for patients with IUGR. No heterogeneity was found in any analysis.

The prevalence of undiagnosed CD has been investigated also in patients with pre-eclampsia by Wolf *et al*. Women with a history of pre-eclampsia were tested for anti-TG and anti-endomysium antibodies seropositivity but an increased incidence of CD compared with controls was not found (Wolf *et al.*, 2008). Because of the few number of studies available, definitive conclusions cannot be found.

With respect to cohort studies, the risks of miscarriage (Fig. 4A), recurrent miscarriage (Fig. 4B), unexplained stillbirth (Fig. 4C), IUGR (Fig. 5A), LBW (Fig. 5B), preterm delivery (Fig. 5C) and pre-eclampsia (Fig. 5D) in celiac patients in comparison with controls was obtained through data combination. The risks of miscarriage, IUGR, LBW and preterm delivery were shown to be significantly higher in celiac patients with RRs of 1.39 (95% CI 1.15–1.67), 1.54 (95% CI 1.22–1.95), 1.75 (95% CI 1.23–2.49) and 1.37 (95% CI 1.19–1.57), respectively. On the contrary, no increased risk of recurrent miscarriage, unexplained stillbirth or pre-eclampsia was found in celiac patients. The heterogeneity was slight with regard to IUGR, LBW and preterm delivery but high in the analysis on recurrent miscarriage. In all those cases, a random-effect model was used.

After stratifying the analyses for current treatment of the disease, it emerged that the risks of IUGR, LBW and preterm delivery were significant higher in untreated patients but not in patients on a GFD (Table I). Finally, Khashan *et al*. investigated the risk in celiac women of delivering SGA babies in a well-designed population-based cohort study, and observed that women with untreated CD delivered smaller babies, showing a higher risk of SGA infants compared with women without CD. Furthermore, they showed that women with CD on a GFD had no increased risk of SGA compared with women without CD, highlighting the cause–effect relationship between exposure to gluten, and then of the activity of CD, and the considered adverse pregnancy outcome (Khashan *et al.*, 2010). Unfortunately, no comparable studies have been found and that is currently the only study supporting this observation.

The main limits of these meta-analyses were the potential for selection bias within each of the studies and the lack of quality assessment of these single studies. Furthermore, the analysis of publication bias by funnel plots was not exhaustive because of the small number of studies for each end-point. Publication bias could only be excluded for the overall occurrence of adverse pregnancy outcomes in the case–control studies (Fig. 2A).

Current hypotheses of CD-induced mechanisms of obstetric failures

The pathogenesis of reproductive disorders in CD is unclear, but some hypotheses have been suggested. Those hypotheses may be classified under two main headings: nutrient deficiency and autoimmune mechanisms.

Malabsorption and nutrient deficiency

Nutrient deficiency, often occurring in active CD, has historically been considered the main cause of gynecologic disorders and adverse pregnancy outcomes associated with the disease. Indeed, the abnormal villous structure of the small intestine, characteristic of CD, generally results in malabsorption and can lead to minor hematologic abnormalities, anemia and other selective nutrient deficiencies, such as zinc, selenium and folic acid (Jameson, 1976; Yüce *et al.*, 2004; Haapalahti *et al.*, 2005; Singhal *et al.*, 2008; Högberg *et al.*, 2009), which play significant roles in pregnancy and fetal development.

Zinc deficiency has been shown to cause impaired synthesis and secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH), which may subsequently cause an abnormal ovarian axis, secondary amenorrhea, spontaneous abortions and pre-eclampsia (Bedwal and Bahuguna, 1994). Selenium deficiency also affects the synthesis and secretion of FSH and LH (Bedwal and Bahuguna, 1994). Finally, it is well recognized that folic acid is an essential vitamin in nucleic acid

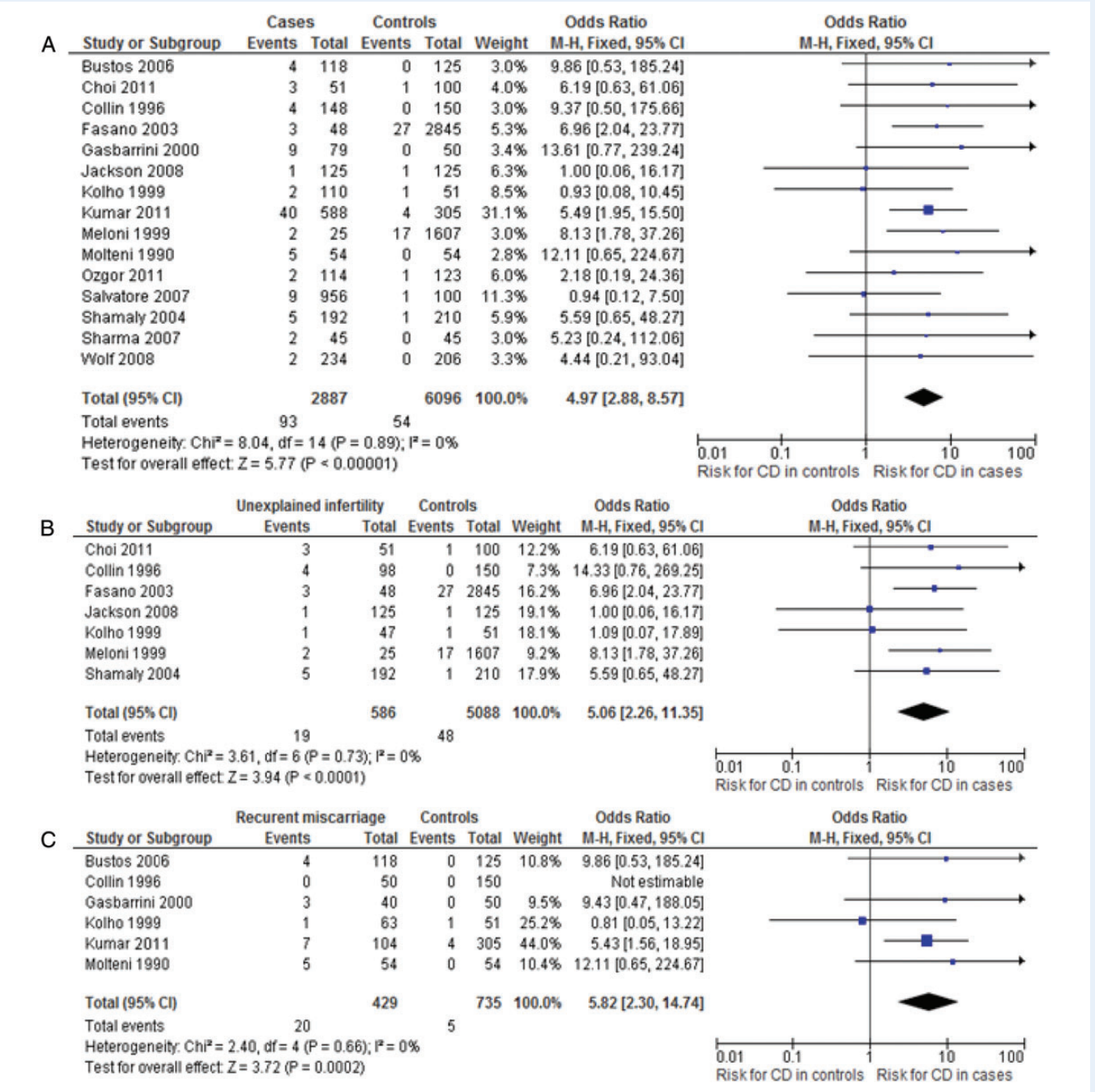

Figure 2 Overall risk of CD in cases of adverse pregnancy outcomes (cases) in comparison with healthy women (controls) ($n = 15$ studies) (**A**). Risk of CD in patients with unexplained infertility ($n = 7$ studies) (**B**) or with history of recurrent miscarriage ($n = 6$ studies) (**C**) in comparison with controls. OR, odds ratio; 95% CI, 95% confidence intervals.

metabolism, and that deficiency of it has an impact on rapidly proliferating tissues, such as the embryo, especially in its neuronal development. However, Dickey *et al*., investigating the possibility that maternal CD might be a risk factor for the occurrence of fetal neural tube (NTD) related to folic acid deficiency, found that the majority of NTD were not associated with maternal CD (Dickey *et al.*, 1996), which means that an increased incidence of CD in this population of a nutrient deficiency-related fetal abnormality could not be confirmed.

Unfortunately, nutritional studies in CD during pregnancy are very limited. Most of studies investigating malabsorption and nutrient deficit in CD have been completed in children, so that, available data do not seem to offer a definitive explanation for reproductive disorders in women with CD.

The nutritional status has been emphasized by Kotze as an important and relevant factor in determining pregnancy outcome. The severity of malnutrition directly correlated with the frequency of gynecologic and

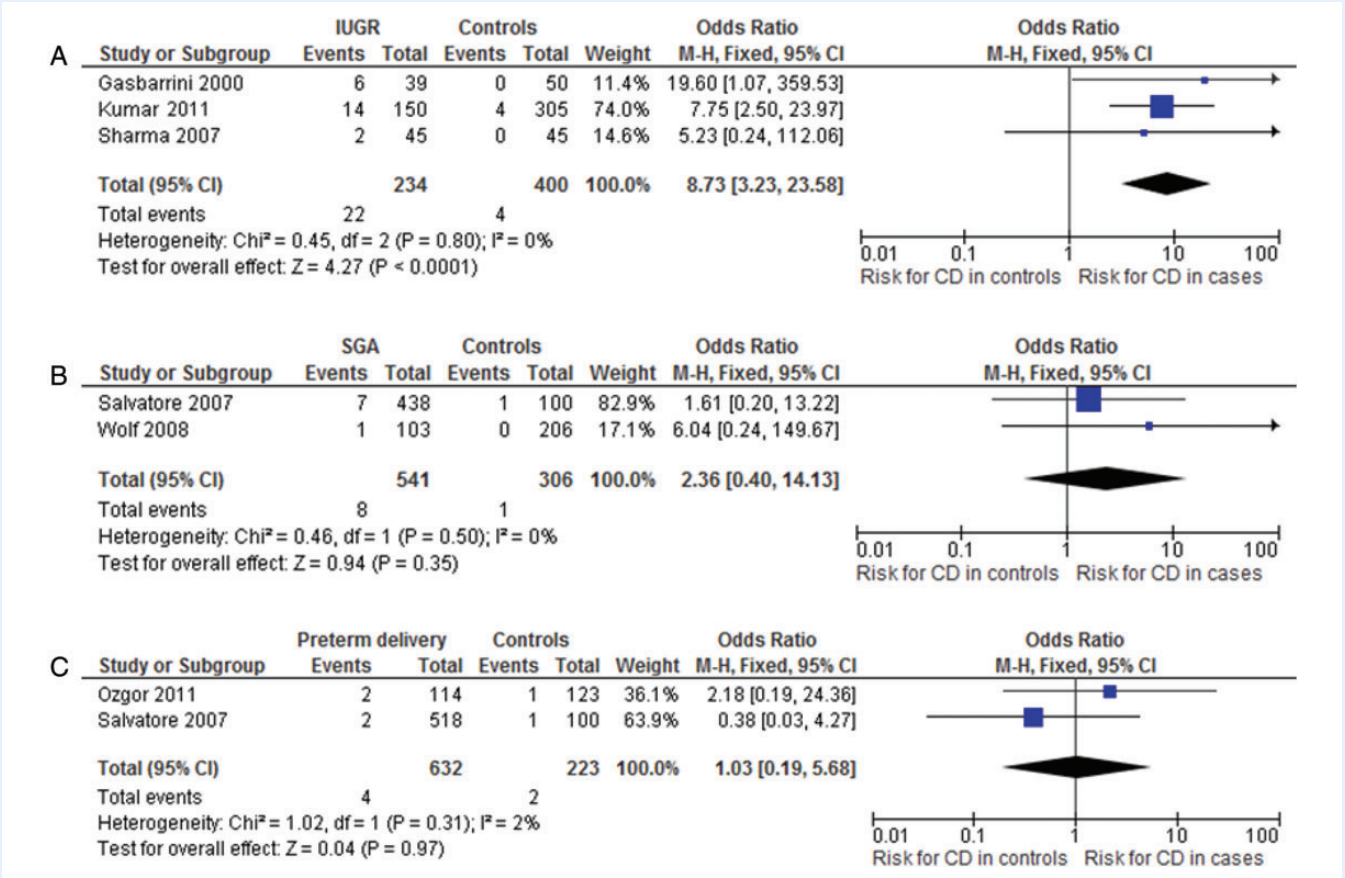

Figure 3 Risk of CD in patients with intrauterine growth restriction (IUGR) (**A**) ($n = 3$ studies), small for gestation age (SGA) babies (**B**) ($n = 2$ studies) or preterm delivery (**C**) ($n = 2$ studies) in comparison with controls. OR, odds ratio; 95% CI, 95% confidence intervals.

obstetric disorders, and, as expected, adherence to a GFD was shown to significantly ameliorate reproductive performances of celiac women (Kotze, 2004). On the other hand, women with infertility associated with total and subtotal villous atrophy were often shown to have neither severe malnutrition nor signs of trace element deficiency (Wilson *et al.*, 1976; Collin *et al.*, 1996; Meloni *et al.*, 1999; Shamaly *et al.*, 2004).

Clearly, the current knowledge does not point to nutrient deficiency as the main pathologic condition responsible for reproductive failures occurring in CD. Further studies are needed to precisely define the role of altered absorption and resultant nutritional changes on female fertility in untreated CD, as well as the effects of a GFD, especially with restoration of normal nutritional status.

Autoimmune mechanisms

Since the increased incidence of obstetrics failures in CD cannot be explained by malabsorption alone, new pathogenic mechanisms have been investigated during recent years to demonstrate a direct immune-mediated impairment of the physiologic processes occurring during embryo implantation and placental development in women with CD.

Anti-TG antibodies induce trophoblast apoptosis

Patients with CD on a gluten-containing diet generally show increased levels of serum autoantibodies, in particular, of anti-TG antibodies (Dieterich *et al.*, 1997; Mäki, 1997). It is has been firstly hypothesized that circulating anti-TG antibodies, produced in active CD, could be not only a diagnostic marker of CD, but also directly involved in placental-related pregnancy complications.

Indeed, it is noteworthy that anti-TG antibodies are not only an epiphenomena in CD, but are also reported to cause the intestinal and neurologic damage, interfering with the cell cycle of human enterocytes and inducing the apoptosis of neuronal cells, respectively (Caputo *et al.*, 2010; Cervio *et al.*, 2007).

The rationale of a possible direct binding of circulating anti-TG antibodies to placental cells *in vivo* is supported by the evidence that the enzyme TG is expressed in many different tissues and organs, and it is found intracelluarly as well as extracellularly. In particular, it has been demonstrated that TG is expressed in endometrial cells as well as in stromal and trophoblast placental cells, with higher levels in late pregnancy (Robinson *et al.*, 2006).

Since TG, in the extracellular environment, is involved in extracellular matrix assembly and cell adhesion, spreading and migration in diverse tissues (Zemskov *et al.*, 2006; Park *et al.*, 2010), it is probable that one or more of the mentioned TG-mediated cellular activities are likely to play a critical role in the implantation process. Thus, TG on syncytiotrophoblasts may be a target of maternal autoantibodies in CD, and in particular, it is conceivable that the binding of circulating anti-TG antibodies to placental cells could be an immunologic mechanism by which CD may interfere with pregnancy outcome.

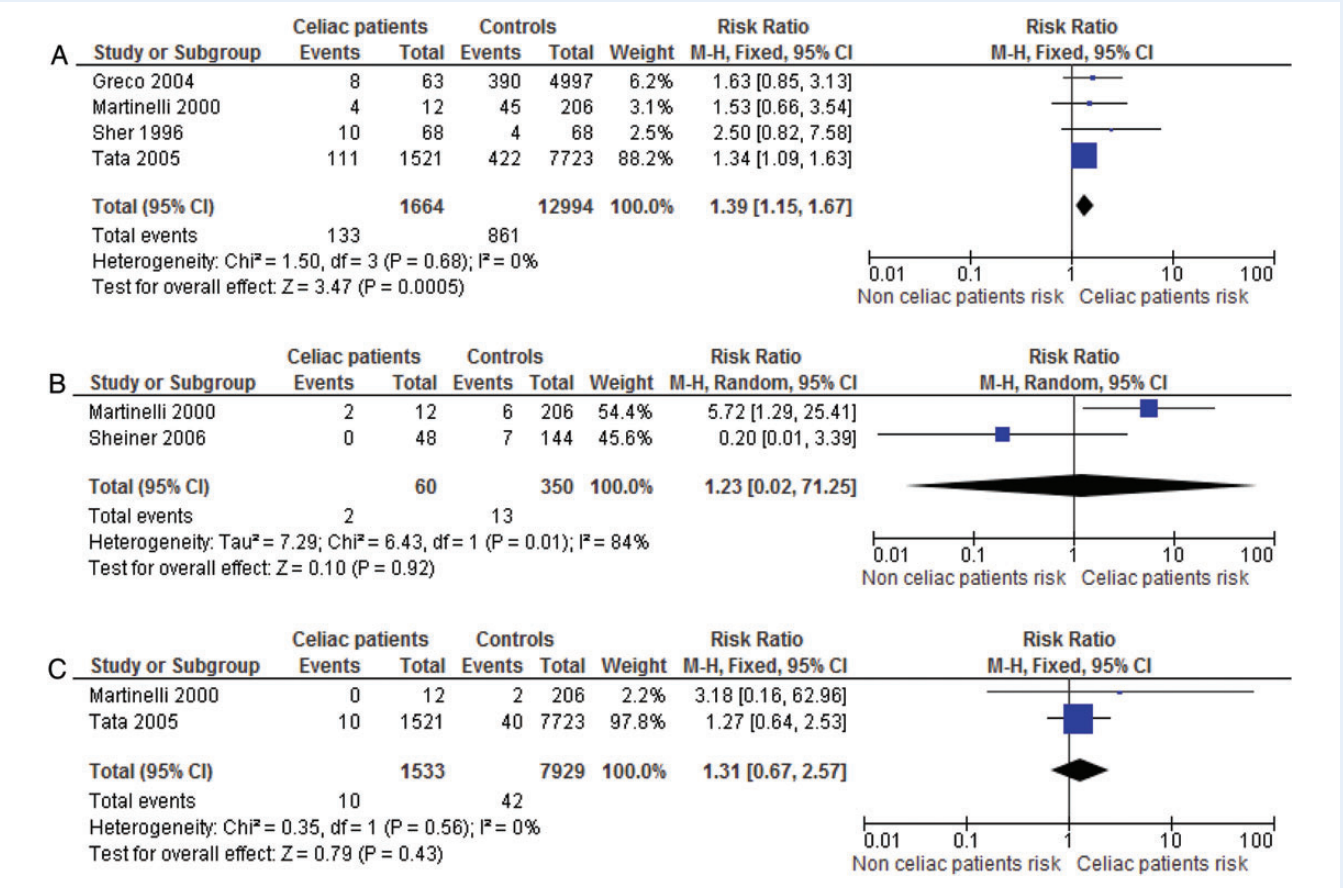

Figure 4 Risk of miscarriage (**A**) ($n = 4$ studies), recurrent miscarriage (**B**) ($n = 2$ studies) or unexplained stillbirth (**C**) ($n = 2$ studies) in celiac patients in comparison with controls. RR, relative risk; 95% CI: 95% confidence intervals.

Normal development and function of the placenta requires invasion of the maternal decidua by extravillous trophoblast (EVT), followed by abundant and organized vascular growth (Helige *et al.*, 2008). EVTs produce large amounts of basic proteins and hormones involved in maintenance of the pregnancy. Therefore, it is likely that increased apoptosis of EVT may contribute to the pathophysiology of human miscarriage and IUGR (Hadziselimovic *et al.*, 2007; Minas *et al.*, 2007), which are characteristic placental-related complications, found at higher frequency in women with active CD than in the general population. Supporting this hypothesis, Hadziselimovic *et al.* (2007) showed increased apoptosis of EVT in placentas of celiac women noncompliant to a GFD, which is consistently linked to the low birthweight of newborns.

Interestingly, Anjum *et al.* have shown that anti-TG antibodies of IgA class are able to directly bind to the syncytial surface of the placenta, significantly inhibiting TG activity (Anjum *et al.*, 2009). Anti-TG antibody-mediated inhibition of syncytial TG is an intriguing hypothesis to explain a functional impairment of placental development. However, Anjum *et al.* only evaluated the effect of IgA class autoantibodies on placental cells and, even if IgA are the class of immunoglobulins secreted at highest concentration in active CD, it is well known that the IgG class is the only one able to cross the placental barrier and to potentially determine a direct effect at the fetal site of the placenta.

Based on these preliminary observations, we hypothesized that anti-TG antibodies could bind to TG expressed on trophoblast cells *in vivo*, determining a functional impairment by affecting the invasive potential.

To assess our hypothesis, we isolated both IgG and IgA polyclonal fraction from sera of patients with active CD, not on a GFD, and with a high titer of anti-TG antibodies (Di Simone *et al.*, 2010). Human primary trophoblasts cells provide a reliable model for studying the molecular mechanisms of the pathologic conditions affecting the placenta (Di Simone *et al.*, 2005). Thus, trophoblast cell cultures were exposed to increasing concentration of IgA and IgG anti-TG antibodies, both commercially available and isolated from celiac women. We specifically focused on the effect of anti-TG antibodies on placental invasiveness, activity of cellular matrix metalloproteases (MMPs) and cellular apoptosis, as indicators of trophoblast damage.

We observed that both the polyclonal fractions of anti-TG antibodies and the commercial monoclonal anti-TG antibody (CUB7402) were able to directly bind to trophoblast cells, and significantly reduce trophoblast invasiveness through apoptotic damage (Di Simone *et al.*, 2010). In addition to the anti-TG antibody binding-mediated increase in trophoblast apoptosis, a significant decrease in MMPs activity was observed in this study, and this could be an indirect effect of the increase in trophoblast apoptosis (Di Simone *et al.*, 2010) (Fig. 6A).

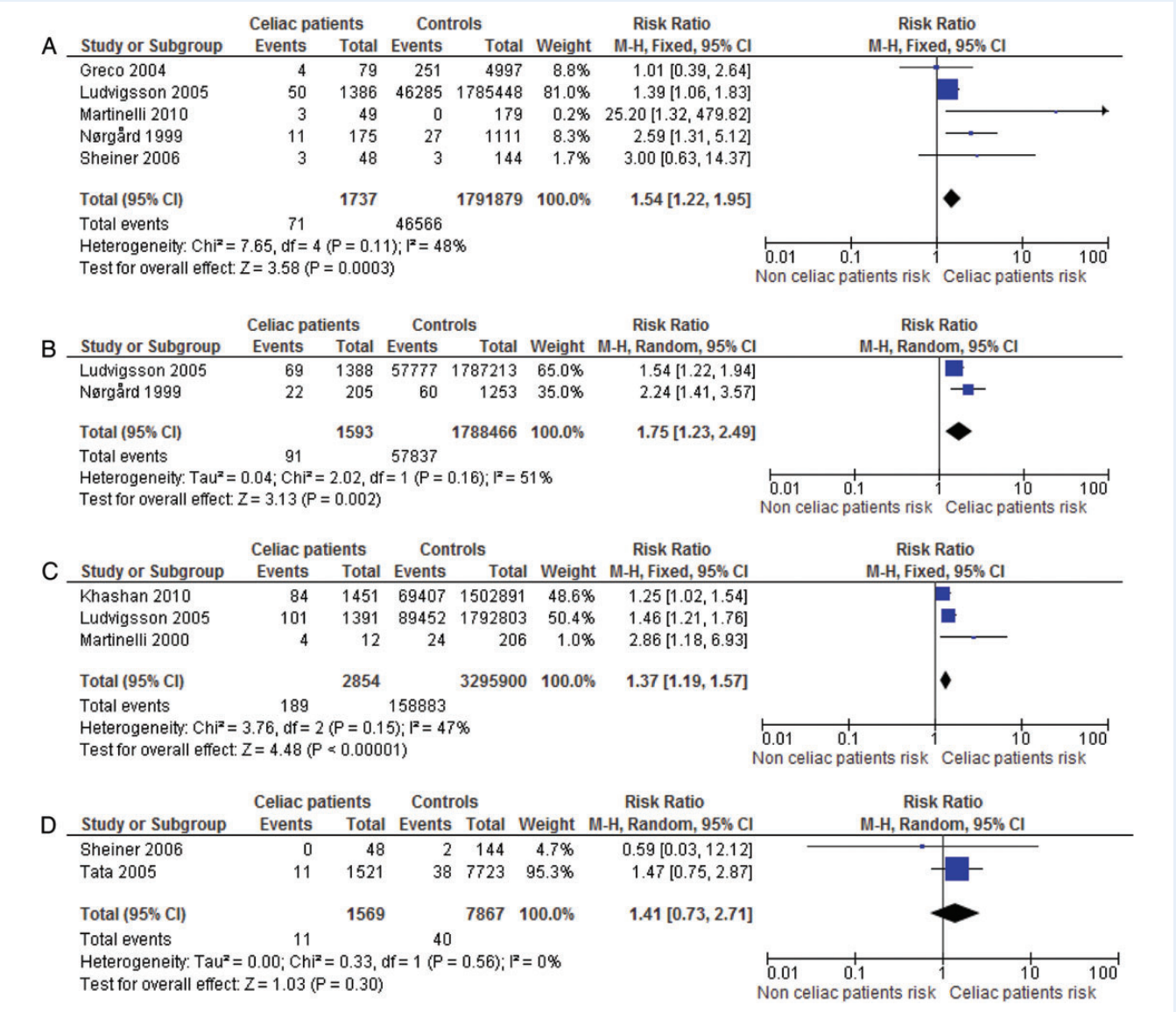

Figure 5 Risk of intrauterine growth restriction (IUGR) (**A**) ($n = 5$ studies), low birthweight (LBW) (**B**) ($n = 2$ studies), preterm delivery (**C**) ($n = 3$ studies) or pre-eclampsia (**D**) ($n = 2$ studies) in celiac patients in comparison with controls. RR, relative risk; 95% CI: 95% confidence intervals.

Table I Risk for IUGR, LBW, preterm delivery in treated and untreated celiac patients in comparison with controls (RR with 95% CI).

	Untreated patients	Treated patients
IUGR (Nørgård *et al.*, 1999; Greco *et al.*, 2004; Ludvigsson *et al.*, 2005)	1.98 (95% CI 1.12–3.52); I^2: 52%	1.28 (95% CI 0.93–1.76); I^2: 0%
LBW (Nørgård *et al.*, 1999; Ludvigsson *et al.*, 2005)	2.47 (95% CI 1.86–3.29); I^2: 0%	1.22 (95% CI 0.91–1.63); I^2: 0%
Preterm delivery (Ludvigsson *et al.*, 2005; Khashan *et al.*, 2010)	1.62 (95% CI 1.05–2.51); I^2: 81%	1.20 (95% CI 0.97–1.48); I^2: 0%

In general, these studies provide a pathogenic model of immune-mediated placental damage potentially occurring *in vivo* in women with active CD and a high titer of circulating anti-TG antibodies, giving a rationale for the increased incidence of placental-related obstetric failures in celiac women.

Anti-trasglutaminase antibodies affect human endometrial angiogenesis

Endometrial angiogenesis and decidualization, as well as trophoblast invasion, are fundamental prerequisites for a successful implantation and a good outcome of pregnancy. In the pregnant uterus, critical angiogenic

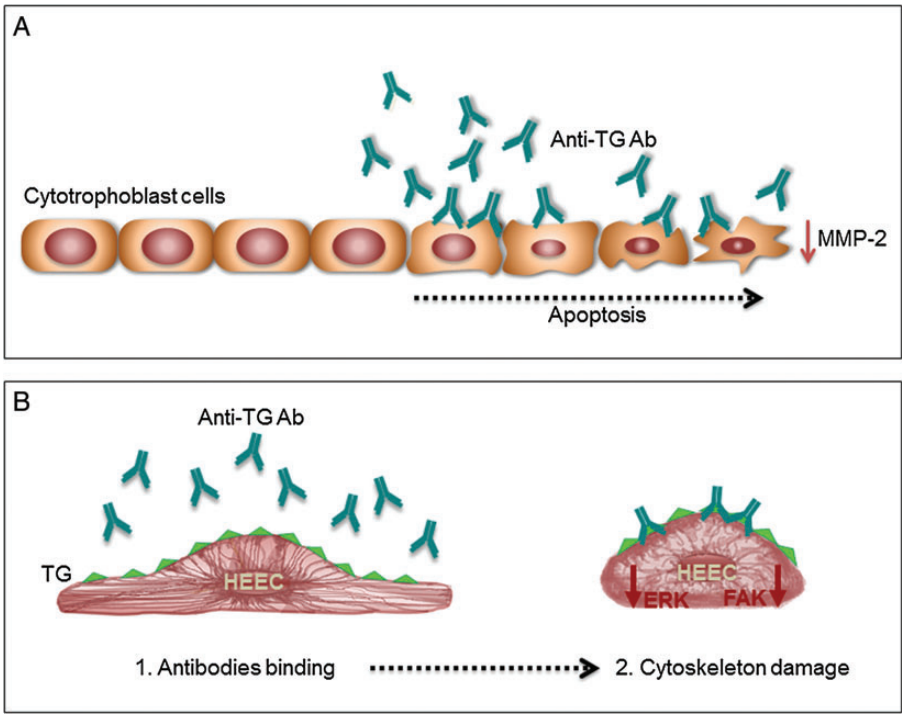

Figure 6 Representative image of the two main mechanisms of anti-TG antibody-mediated placental damage proposed. Anti-TG antibodies from maternal blood circulation bind to trophoblast cells inducing an apoptotic damage (**A**). At the maternal site, anti-TG antibodies binding to endometrial endothelial cells (HEEC) may cause a dramatic disarrangement of the F-actin cytoskeleton impairing the angiogenic process (**B**). MMP, matrix metalloproteinase.

signals are likely to be produced by the decidualizing endometrial cells acting on the endothelial cells to promote their proliferation and differentiation. After stimulation by angiogenic factors, the basement membrane is degraded by MMPs and the proteolytic enzymes secreted by endothelial cells. Then the cells invade, migrate and proliferate into the underlying interstitial matrix and form new capillary structures (Taylor *et al.*, 1992; Murray and Lessey, 1999). Thus, angiogenesis induces fundamental changes in the endometrium, enabling it to accept the blastocyst and initiate the process of implantation.

We hypothesized that, together with a direct apoptotic damage to trophoblast cells, circulating anti-TG antibodies could be responsible for an additional mechanism of impairment of placental development, this time at the maternal site of the placenta: endometrial angiogenesis. To assess this hypothesis, we firstly isolated human endometrial endothelial cells (HEECs) from placental explants through immune selection and put them in culture. After incubation of HEEC cultures with both IgA and IgG polyclonal immunoglobulins isolated from the sera of celiac patients, and with commercial monoclonal anti-TG IgG (CUB 7402), we demonstrated a direct binding of the anti-TG antibodies to cell membrane of HEECs and a consequent decrease in cellular TG activity (Di Simone *et al.*, 2013). This binding was followed by a striking decrease of *in vitro* angiogenesis, in terms of the number and total length of capillary-like tubes formed by HEECs (Di Simone *et al.*, 2013). To confirm this observation *in vivo*, we also evaluated the effect of anti-TG antibodies in a murine model of angiogenesis, confirming the autoantibody-mediated inhibiting effect obtained *in vitro* (Di Simone *et al.*, 2013).

The specific role of cellular TG as target of anti-TG antibodies was defined by investigating the effect of its down-regulation by siRNA on this mechanism of inhibition. We found that treatment of TG-silenced HEEC with polyclonal or monoclonal anti-TG antibodies did not cause a reduction in cell differentiation in contrast with HEECs with normal expression of TG, confirming the supposed role of this enzyme expressed on HEEC membranes as a target for the anti-TG antibodies (Di Simone *et al.*, 2013).

To identify the molecular mechanisms involved in the inhibition of angiogenesis, we evaluated the activity of MMP-2 in HEEC culture in the presence of polyclonal and monoclonal anti-TG2 antibodies and observed a significant reduction of both pro- and active MMP-2 protein levels. Thus, it is likely that, among the possible molecular mechanisms responsible for the anti-TG antibody-induced angiogenesis inhibition is a reduction of MMP secretion and of extracellular matrix degradation. Surprisingly, the functional impairment of HEEC angiogenesis was not associated with an increase in cell apoptosis. In addition, having not found a reduction of vascular endothelial growth factor secretion in the medium of HEEC cultures after anti-TG antibody binding, we also supposed that a mechanism other than cell apoptosis or decreased pro-angiogenic factors secretion could be involved in this antibody-mediated angiogenesis inhibition.

Thus, since a negative effect of anti-TG antibodies on human umbilical vein endothelial cell (HUVEC) cytoskeleton organization has been shown (Myrsky *et al.*, 2008), we hypothesized that anti-TG antibodies, by binding to TG on cells surface, may exert their effects interacting with actin fibers of cytoskeleton, closely connected with cell membranes.

Indeed, it is well known that the cytoskeleton mediates a variety of cellular functions, such as cell–substrate and cell–cell adhesion, which is fundamental for cellular replication and migration, and that a proper actin dynamics and cytoskeleton rearrangement of endothelial cells are recognized to play a pivotal role in the angiogenic process. Hence, we investigated whether a specific autoantibody-induced impairment of HEEC F-actin fiberes organization might explain the inhibited angiogenesis observed after anti-TG antibody exposure in the *in vitro* and *in vivo* models.

We documented a dramatic disarrangement of the F-actin cytoskeleton in anti-TG antibodies-treated HEEC both directly, by visualization through confocal microscopy, and indirectly, by detecting an increase in the cytoskeleton stiffness and a reduction of fluidity and cell adhesiviness of HEEC membranes, which represents the functional counterpart of cytoskeleton modification. Since membrane fluidity reflects the structure of lipids in the membrane, while adhesiviness strongly depends on the cytoskeleton architecture, our results shed light on a functional interplay between membrane lipids and the cytoskeleton (Khurana, 2000; Chichili and Rodgers, 2009) (Fig. 6B).

To better understand the intracellular mechanisms regulating the changes in TG-mediated HEEC motility and cytoskeletal organization during the process of angiogenesis, we also examined the effect of anti-TG antibodies on FAK and ERK activation, the key kinases of the intracellular pathway regulating cytoskeleton arrangement and the transcription of pro-angiogenic factors (Okajima and Thorgeirsson, 2000; Huang *et al.*, 2004). The activation of FAK results in phosphorylation of downstream target molecules, including ERK, which are localized at focal adhesion sites, thus ensuring cell contact with the extracellular matrix and providing the structural links between the extracellular matrix and polymerized actin filaments involved in focal adhesion, cell shape and motility (Hanks and Polte, 1997; Weisberg *et al.*, 1997; Small *et al.*, 2002). Furthermore, angiogenic factors directly stimulate angiogenesis by activating the ERK- and FAK-dependent signaling pathways and the inhibition of these cascade suppresses their angiogenic activities (Lee *et al.*, 2006; Chung *et al.*, 2009; Namkoong *et al.*, 2009). We demonstrated that anti-TG antibodies inhibited the intracellular pro-angiogenic signal mediators ERK and FAK, providing an additional molecular mechanism by which anti-TG antibodies may cause their anti-angiogenic activity on endometrial cells.

In conclusion, antigenic structures suitable for anti-TG antibodies are present on HEEC and the binding of autoantibodies to endometrial endothelial cells and their consequent functional inhibition might represent a key mechanism by which anti-TG antibodies could affect embryo implantation and placentation at the maternal site of the fetal–maternal unit. Because endometrial angiogenesis is essential for placental development and fetal growth, this could be considered a novel pathogenic mechanism contributing to the adverse pregnancy outcomes occurring in CD.

Conclusions

CD is the most common autoimmune disease with a prevalence of 1% in the general population worldwide. Our meta-analysis demonstrates that patients with unexplained infertility, recurrent miscarriage or IUGR have a nearly 5-, 6- or 8-fold, respectively, increased risk of being affected from CD compared with the general population. Often, patients with the above reproductive disorders have no overt symptoms of CD, or at most, fatigue associated with iron deficiency anemia. As a result, irregular menstruation, reduced fertility and/or adverse pregnancy outcomes may be the initial clinical feature that ultimately results in a diagnosis of CD.

Thus, a serologic screening for CD, performed by testing the patients' sera for endomysial and anti-TG antibodies is strongly suggested in cases of unexplained infertility, recurrent miscarriage and IUGR. However, not enough evidence is available to recommend a screening for CD to women with a history of SGA, preterm birth or pre-eclampsia.

We also observed that celiac women have a significantly higher risk of experiencing miscarriage (RR 1.39–95% CI 1.15–1.67), IUGR (RR 1.54–95% CI 1.22–1.95), LBW (RR 1.75–95% CI 1.23–2.49) or preterm delivery (RR 1.37–95% CI 1.19–1.57) compared with healthy women. However, no significantly increased risk of recurrent miscarriage, unexplained stillbirth or pre-eclampsia was found in celiac patients.

Before–after studies have shown that the risk for IUGR, LBW and preterm delivery in celiac women was significantly reduced by adherence to a GFD. As a consequence, it is mandatory for physicians to make celiac women aware of the potential negative effects of active CD disease, also in terms of reproductive performances, and of the importance of a strict GFD to ameliorate their general and reproductive health.

Concerning the pathogenic mechanisms indicated to be involved in adverse pregnancy outcomes occurring in CD, we believe that the close link between CD activity and a higher risk of reproductive failures may strongly suggest a central role of the immune system in causing obstetric failures. *In vitro* studies have provided two main pathogenic models of placental damage at the feto-maternal interface. On the embryonic side of the placenta, a direct binding of anti-TG antibodies to trophoblast cells, with a reduction in trophoblast invasiveness due to apoptotic damage, has been proposed. Furthermore, on the maternal side of the placenta, anti-TG antibodies may also be detrimental to endometrial angiogenesis by impairing the cytoskeleton structure in endometrial endothelial cells.

In aggregate, these studies support a role of gluten exposure in eliciting immune responses likely responsible for the occurrence of some pregnancy complications.

Further studies are required to provide more details about the complex interference of this autoimmune disease in human reproduction.

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Authors' roles

C.T., A.F., N.D.S., M.D.S., A.G. and G.S. were responsible for the study concept, design and supervision. C.T. and C.d.W. performed the literature searches and extraction of data. N.D.S., R.C., A.F. and C.T. conducted the basic research experiments. N.D.S., C.T., A.F. and C.d.W. were responsible for the analysis and interpretation of the data. C.T., A.F., C.d.W. and N.D.S. drafted the manuscript.

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Conflict of interest

There are no competing interests.

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