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Short communication

Human leukocyte antigen (HLA) DQ2/DQ8 prevalence in recurrent pregnancy loss women

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ABSTRACT

Objective: Over the last few years, medical scholars have reported the significant association between recurrent pregnancy loss (RPL) and celiac disease (CD). Various pathogenic mechanisms underlying the pregnancy failure in CD have been suggested: among them the ability of anti-transglutaminase antibodies to impair the trophoblast invasiveness and endometrial endothelial cells differentiation and disrupt early placentation. CD shows a complex non-Mendelian pattern of inheritance, involving major histocompatibility complex (MHC) genes. The strongest effects are mapped to the classical human leukocyte antigen (HLA)-DQA1 and HLA-DQB1 genes. Specifically, the common haplotypes DQ2.5, DQ2.2, and DQ8 have been shown to increase CD risk by six-fold on average. MHC region contains genes with immunological functions and is responsible for the strongest association signals observed in most immune-mediated diseases. The aim of our study was to investigate the prevalence of the HLA-DQ2/DQ8 haplotypes in RPL, outside of CD.
Methods: The study population included women with history of RPL (≥3 spontaneous pregnancy losses) and women with at least two previous uncomplicated term pregnancies (control group, CTR). All women gave their informed consent to use their data for research purposes.
Results: 97 RPL women and 55 CTR were considered in the study. Mean age of the RPL sample was 37.7 (standard deviation, SD, 3.0; min 27; max 39). Mean age of the control group was 35.6 (SD 3.0; min 26 years; m, max 38). A significantly increased prevalence of HLA-DQ2/DQ8 haplotype positivity was found in RPL population compared to control women (52.6% vs 23.6%; $p < 0.01$).
Conclusions: Our observations show for the first time a higher proportion of individuals HLA DQ2/DQ8 positive in women with RPL as compared to controls (and to general population estimates). Further studies are needed to better understand (i) the possible pathogenic mechanism to this observation; (ii) the clinical and therapeutic implications of our observation in order to provide a new approach to RPL couples.

1. Introduction

Recurrent pregnancy loss (RPL) is historically defined as three or more subsequent spontaneous pregnancy losses [1]. In 2012, the American Society for Reproductive Medicine Practice Committee formalized the definition slightly differently—the Society defined RPL as "the subsequent occurrence of two or more clinically (i.e., pregnancies documented by ultrasonography or histopathological examination) failed pregnancies" [2]. This difference may have implications mainly for clinical research studies rather than for clinical practice. Indeed, the risk of another pregnancy loss after two miscarriages is only slightly lower than that of women with three or more spontaneous abortions (24%–29% vs 31%–33%); furthermore, no significant difference has been found when examining test results related to two or more prior losses [3,4].

RPL occurs in about 2–3% of clinically diagnosed pregnancies of reproductive-aged women. At present, accepted etiologies for RPL include parental chromosomal abnormalities, untreated hypothyroidism, uncontrolled diabetes mellitus, certain uterine anatomic abnormalities, antiphospholipid antibody syndrome, heritable and/or acquired thrombophilias, infections, and environmental factors [5–10]. In addition, an increased risk of auto- and cellular immune abnormalities, such as an increased positivity for anti-nuclear (ANA) and/or -thyroid antibodies

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have been observed in RPL women [5,11–13]. After evaluation for these causes, approximately 40% of all cases of RPL lack of an associated factor. This group of cases represents, to date, the challenge for researchers.

Over the last few years, medical scholars have examined the relationship between RPL and celiac disease (CD), one of the most common inflammatory disorders of the small intestine affecting up to 1% of individuals in Western populations [14–16]. In particular, our recent meta-analysis has reported an odds ratio (OR) value for CD of 5.82 (95% CI 2.30–14.74) for women experiencing recurrent miscarriage as well as a higher risk of miscarriage in celiac patients, bearing a relative risk (RR) of 1.39 (95% CI 1.15–1.67) [14]. The pathogenic mechanism underlying the pregnancy failure in CD is not well understood. Beyond the nutrient deficiency, which can be found in celiac patients and characterized by the lack of elements like zinc, selenium, and folic acid, the ability of anti-transglutaminase antibodies to impair trophoblast invasiveness and endometrial endothelial cells differentiation has been suggested as a possible disruptor of early placentation [15,16].

In celiac patients beyond the ingestion of gluten, which certainly represents the environmental trigger, the genetic predisposition strongly contributes to the development of the disease [17,18]. Studies on siblings confirmed a concordance of around 80% in monozygotic twins, less than 20% among dizygotic twins, and a risk for a patient's siblings to develop a CD around 20–60, as confirmed by familial aggregation studies [19–23]. The major histocompatibility complex (MHC) gene region is the main genetic factor in the disease development, with the strongest effects mapped to the classical human leukocyte antigen (HLA)-DQA1 and HLA-DQB1 genes. Specifically, the common haplotypes DQ2.5, DQ2.2, and DQ8 have been shown to increase disease risk by six-fold on average [24–26]. Approximately 25% of the general Caucasian population is HLA-DQ2/DQ8. From these genetically susceptible individuals, only 4% develop CD [16,27].

Given these premises, we aimed at studying the prevalence of HLA-DQ2/DQ8 positivity in RPL women, outside of CD. The observation of a possible correlation between HLA-DQ2/DQ8 haplotype positivity and RPL, if confirmed by further studies, including larger number of women, might suggest new diagnostic and therapeutical approaches for RPL women.

2. Materials and methods

2.1. Patients

The study population included women with history of RPL and women with previous uncomplicated term pregnancies (control group). RPL and control group women were recruited over a period of 6 months (from January to June 2015) at the Recurrent Pregnancy Loss Outpatient Clinic and the Gynecology Outpatient Clinic, respectively, at the Department of Obstetrics and Gynecology, A. Gemelli Universitary Hospital, Rome, Italy. RPL women have had three or more subsequent early (<10 weeks of gestation) spontaneous pregnancy losses clinically documented by ultrasonography and/or histopathology examination. Control group included women with at least two previous uncomplicated term pregnancies. The inclusion criteria for both groups were as follows: Caucasian, age < 39 years, healthy, regular ovulatory cycles (28–32 days), normal endocrine profile, normal serum levels of follicle-stimulating hormone (FSH < 10 mIU/ml), luteinizing hormone (LH < 10 mIU/ml), and anti-mullerian hormone (AMH > 2 ng/ml) on day 3 of the menstrual cycle, normal serum levels of total immunoglobulin (Ig) G and IgA, absence of gastrointestinal symptoms, absence of a prior diagnosis of CD [28]. In order to investigate the presence of a possible association between the HLA status and the presence of antibody positivity, the anti-thyroid (-thyroglobulin/-thyroid peroxidase), -cardiolipin, -β2 glicoprotein I (β2GPI), -prothrombin, -nuclear, -transglutaminase, -endomysium, and -gliadin antibody positivity was also performed. The autoantibody positivity was confirmed on two or more occasions at least 12 weeks apart. Screening for autoimmunity was performed after at least 8 weeks apart the last miscarriage.

The presence of thrombophilic defects including activated protein C resistance/factor V G1691A mutation, prothrombin G20210A mutation, protein C, protein S and/or antithrombin III deficiency, lupus anticoagulant, hyperomocisteinemia was recorded.

All women gave their informed consent to use, anonymously, their data for research purposes, and the protocol was approved by the ethics committee of A. Gemelli Universitary Hospital, Università Cattolica del Sacro Cuore, Rome, Italy.

2.2. Sample collection and analysis

Peripheral blood was collected in ethylene-diamino-tetra-acetic (EDTA) tubes from RPL and control women. HLA-DQ2/DQ8 analysis was performed, after amplification of human DNA isolated from peripheral blood, by real-time polymerase chain reaction following the manufacturer's instructions (XeliGen RT, Eurospital SpA, Italy). Briefly, PCR with sequence-specific primer tested for the following alleles: DQA1*01, DQA1*0201, DQA1*03, DQA1*05, DQA1*06, DQB1*02, DQB1*0301/03, DQB1*0301/04, DQB1*0302, DQB1*0305, and DQB1*04. The following DR alleles were typed in order to determine the presence of DQ/DR haplotypes: DRB1*03, DRB1*04, DRB1*07, DRB1*11. DQ2 positivity was defined as DQA1*05 in cohort with DQB2*02 (DQ2.5), or DQA1*0201 (DQ2.2)/DQA1*03 (DQ2.3) with B1*02. DQ8 positivity was defined as DQA1*03 with DQB1*0302.

Anti-thyroglobulin and -thyroperoxidase antibodies were tested by chemiluminescence immunoassay (CMIA method, ADVIA Centaur XP Siemens Healthcare, Italy). Anti-cardiolipin and -β2GPI antibodies were tested by using a specific chemiluminescence immunoassay (Zenit Autoimmunity, Menarini Diagnostics, Italy). Screening for ANA was performed by indirect immunofluorescence assay using a commercially available kit (Eurospital SpA, Italy).

2.3. Statistical analysis

Means and standard deviation (SD) were used to describe quantitative variables whereas absolute and relative frequencies were employed for categorical ones. Pearson chi-square and Fisher's exact tests were used to compare HLA DQ2/DQ8 positivity in patients and healthy controls and to evaluate the association between HLA DQ2/DQ8 and antibody positivity in patients with >3 recurrent pregnancy loss.

3. Results

One hundred four women with RPL history and fifty-five control women were recruited from the RPL Outpatient Clinic and the Gynecology Outpatient Clinic, A. Gemelli Universitary Hospital, respectively. Women showing a repeated positivity (on two or more occasions at least 4 weeks apart) for anti-transglutaminase, -endomisium, and -gliadin antibody were sent to gastroenterologist and subsequent biopsy: 7 RPL women (6.7%) were newly diagnosed with CD [17,18], excluded from the study and, after sending to gastroenterologist, treated with gluten-free diet. No newly diagnosed cases of CD were found in the control group. Mean age of RPL women (n = 97) was 37.7 years of age (minimum 27 years, maximum 39 years, SD = 3.0). Mean age of control group was 35.6 years of age (minimum 26 years, maximum 39 years, SD = 3.0; Table 1).

HLA DQ2/DQ8 positivity was found in 52.6% out of 97 RPL women and in 23.6% out of 55 controls ($p < 0.01$; Fig. 1). The odds ratio for HLA DQ2/DQ8 was 3.6 for patients in respect to controls (IC 95%; 1.71–7.65).

The percentage of patients having positivity for HLA DQ2/DQ8, anti –thyroglobulin/thyroid peroxidase (n = 27; 27.8%), -nuclear (n = 39; 40.2%), -cardiolipin (n = 9; 9.2%), -β2GPI (n = 9; 9.2%)

Table 1
Characteristics of women included in the study.

Characteristics of women	Control group (n = 55)	RPL group (n = 97)	P value
Age (years) ± SD	35.6 (26–39)	37.7 (27–39)	Ns
BMI (kg/m^2)	24.1 (19.31–29.5)	24.72 (17.96–26.23)	Ns
Smoking status, n (%)	25 (45.4%)	20 (20.6%)	<0.05*
Primary aborters (n; %)	Na	65 (67.0%)	–
Secondary aborters (n; %)	Na	32 (33%)	–
Three previous miscarriages (%)	Na	44 (45.3%)	–
Four previous miscarriages (%)	Na	37 (38.1%)	–
Five previous miscarriages (%)	Na	16 (16.5%)	–

* Statistical significance: p < 0.05.

and -prothrombin (n = 8; 8.2%) was recorded. No statistically significant association was observed between anti-thyroglobulin, -thyroid peroxidase, -cardiolipin, -β2GPI, -prothrombin antibody positivity and HLA DQ2/DQ8 status. Statistically significant association was observed between ANA and HLA DQ2/DQ8 positivity. In particular, HLA DQ2-DQ8 positive women showed a 4.1 times higher probability of having ANA positivity (55.3%) with respect to HLA DQ2/DQ8 negative women (23.3%; odds ratio 4.1; IC 95% [1.63–10.38]) (Table 2). HLA DQ2/DQ8 positive women sent to gastroenterologist and informed of the significance of the HLA-DQ2/DQ8 positive result associated to an increased "susceptibility to CD" [17,18].

Table 2
Analysis of association between anti-thyroid, -cardiolipin, -nuclear, β2 glycoprotein 1, -protrombin antibody positivity, and HLA DQ2/DQ8 status.

	HLA DQ2/DQ8 positive	HLA DQ2/DQ8 negative	p
Anti-thyroid antibody			
Positive	15 (31.9%)	11 (23.9%)	0.39
Negative	32 (68.1%)	35 (76.1%)	
Anti-nuclear antibody			
Positive	26 (55.3%)	10 (23.3%)	<**0.01***
Negative	21 (44.7%)	33 (76.7%)	
Anti-cardiolipin antibody			
Positive	4 (9.3%)	3 (7.3%)	1.00[ξ]
Negative	39 (90.7%)	38 (92.7%)	
Anti-β2 glycoprotein 1 Antibody			
Positive	5 (12.2%)	2 (5.1%)	0.43[ξ]
Negative	36 (87.8%)	37 (94.9%)	
Anti-prothrombin antibody			
Positive	1 (2.7%)	5 (14.3%)	0.10[ξ]
Negative	36 (97.3%)	30 (85.7%)	

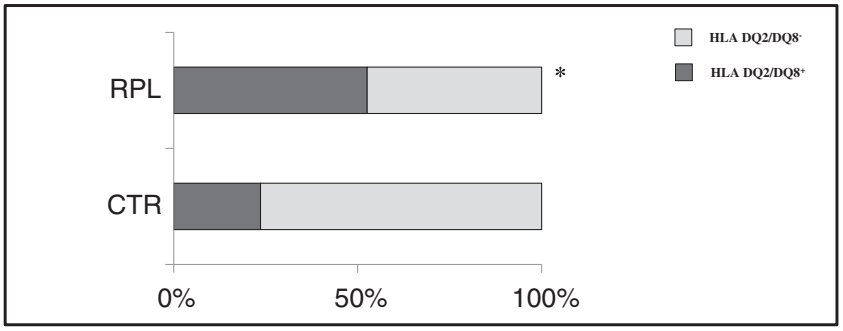
[ξ] Fisher's exact test.
* Statistical significance: p < 0.05.

4. Discussion

In the present study, we observed a significantly increased prevalence of HLA-DQ2/DQ8 positivity in RPL population compared to control women. HLA DQ2/DQ8 haplotypes codify for the DQ2/DQ8 proteins, HLA class II molecules with an important role in the presentation of disease-related peptides to T lymphocytes [29]. In particular, in celiac patients, both proteins are responsible for the presentation of immunogenic gluten peptides to DQ2/DQ8-restricted CD4+ T cells. Once activated, CD4+ T enhance a complex immune response that involves both the innate and the adaptive immune system, with increased production of interferon (IFN) γ, tumor necrosis factor (TNF) α, and autoantibodies like anti-transglutaminase, -endomysium, and -gliadin antibodies; this process will eventually determine the disruption of tight junctions in the intestinal epithelium [29–31] (Fig. 2). In parallel, gluten peptides are able to induce the expression of interleukin (IL) 15, which promotes the infiltration of CD8+ intraepithelial lymphocytes (IELs). Both these mechanisms—epithelial tight junction disruption and apoptosis—lead to the villous atrophy and also render the epithelium more permeable, thereby facilitating access of gluten and propagation of the disease. In the continued presence of dietary gluten, a state of chronic inflammation persists [29–32].

According to a recent meta-analysis, combining data from case–control and cohort studies, women with RPL have a nearly 6-fold increased risk of being affected from CD compared with the general population (OR 5.82, 95% CI 2.30–14.74). Conversely, celiac patients show an increased risk of miscarriage with an RR of 1.39 (95% CI 1.15–1.67) [14]. Several pathogenic mechanisms have been advocated to explain the poor pregnancy outcome. Among them the ability of anti-transglutaminase IgG isolated from sera of untreated celiac patients to bind with both the trophoblast and the endometrial endothelial cells, which represent the fetal and the maternal side of the human placenta, respectively. Consequently, they impair trophoblast invasiveness, through an apoptotic mechanism, and inhibit angiogenesis, by disrupting the F-actin cytoskeleton and the membrane fluidity/adhesiveness/migration [33,34].

Several lines of evidence suggest that the imbalanced response between pro-inflammatory and anti-inflammatory cytokines during early pregnancy may constitute a possible mechanism contributing to the pathogenesis of spontaneous abortion. Indeed abnormal activation of endometrial innate immunity by means of inflammosome, increased



	CTR (n=55)	RPL (n=97)	P value
HLA DQ2/DQ8+ n (%)	13 (**23.6%**)	51 (**52.6%**)	<0.01

Fig. 1. Prevalence of HLA-DQ2/DQ8 positivity in Recurrent Pregnancy Loss (RPL) and control (CTR) women. *Statistical significance: p <0.05.

levels of pro-inflammatory molecules like TNF-α, INF-γ, interleukin (IL) 6, IL-10, and inflammatory leukocytes are reported in women with RPL compared to women with normal pregnancy [35-38]. Given the ability of the dietary gluten to favor a chronic intestinal inflammation, it could be suggested that such a condition might favor a systemic inflammatory state unfavorable during early pregnancy. In addition, RPL is associated with certain autoimmune diseases, most notably antiphospholipid syndrome, CD, ANA, and/or anti-thyroid antibodies positivity [5,11–13, 39-49]. Again, this suggests that abnormalities in the maternal immune response may negatively impact the pregnancy outcome. However, the mechanisms linking immune disorders to RPL remain obscure.

It is now accepted that the alleles encoding HLA-DQ2, specifically, HLA-DQ2.5 (DQA1*05 and DQB1*02) are expressed in around 90% of CD patients. Most of the remaining patients (without HLA-DQ2.5) carry the alleles encoding HLA-DQ8 molecules (DQA1*03 and DQB1*03:02 alleles) [50-52]. In almost all CD patients who carry neither HLA-DQ2.5 nor HLA-DQ8, one of the two alleles encoding HLA-DQ2.5 is present: most commonly DQB1*02 (HLA-DQ2.2) and in a minority of cases, DQA1*05 (HLADQ7.5) [51]. The genetic risk conferred by these described MHC variants ranges from the highest influence of HLA-DQ2.5 to the "apparent" nil effect assigned to HLA-DQ7.5 [24]. CD development in HLA-DQ2- and -DQ8-negative individuals is extremely rare [17,53].

Nevertheless, the HLA-DQ2/DQ8 positivity is a necessary but not sufficient condition for the development of CD. Indeed, even if HLA-DQ2/DQ8 genotype is present in roughly 25–30% of the caucasian individuals, only ~4% of them develop CD [50-52]. In clinical practice, this susceptibility test is important mainly for its high negative predictive value (close to 100%) since the absence of the above-listed HLA molecules makes the onset of CD unlikely [55,56]. Furthermore, it is considered a non-invasive method in those cases of ambiguous findings from intestinal mucosa, of negative serum antibodies or when the patient has started a gluten-free diet before the diagnosis of CD is confirmed.

Recent insights are showing that the most of the chromosome regions associated with CD predisposition contain genes with immunological functions and are responsible for the strongest association signals observed in most immune-mediated diseases [17,57, 58]. For example, DQ2/DQ8 have also been associated with diseases such as insulin-dependent diabetes mellitus and Hashimoto's thyroiditis [59, 60]. This evidence suggests that certain chromosomal regions may confer predisposition to multiple immune-related disorders, thereby

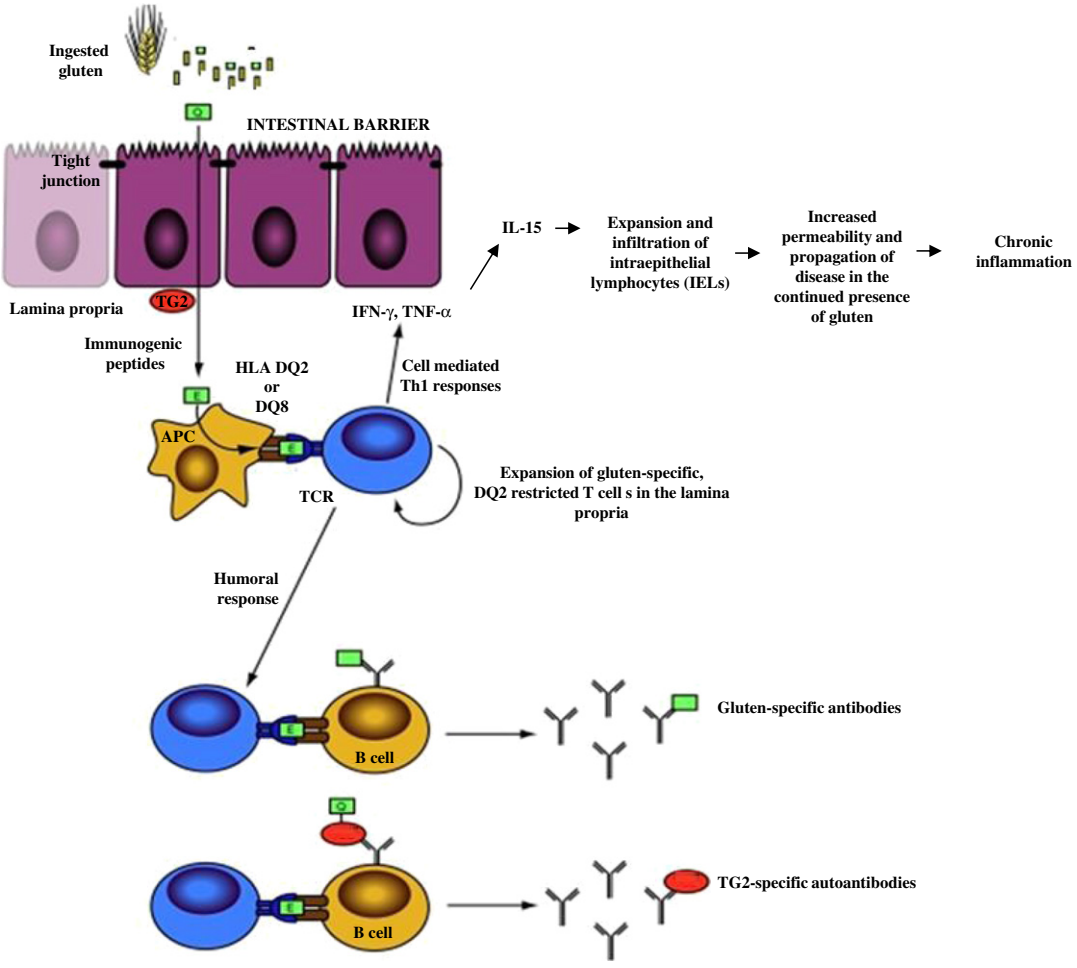


Fig. 2. *Adaptive and innate immune responses to gluten in celiac disease (Mofidied by Ref. 32).* In celiac patients, after digestion of gluten, immunogenic gluten peptides access the lamina propria of intestinal barrier. Subsequently they are deamidated by tranglutaminase 2 (TG2) and loaded onto Human Leucocyte Antigen (HLA) DQ2 or DQ8 proteins. This binding enables the transclocation of the antigen on the surface of antigen-presenting cells (APC) and its presentation to gluten-specific, DQ2/DQ8-restricted CD4+ T cells, causing their activation and clonal expansion. Activated T cells finally mediate the humoral response (leading to the B lymphocyte response and the production of autoantibodies like anti-endomysial, -transglutaminase and -gliadin antibodies) as well as the cell-mediated Th1 response, which, through the secretion of pro-inflammatory cytokines such as Interferon- γ (IFN-γ) and Tumor Necrosis Factor-α (TNF-α), disrupts intestinal tight junction integrity. In parallel, innate peptides act through unknown mechanisms as a stress signal toward enterocytes, inducing expression of Interleukin-15 (IL-15). IL-15 both promotes the infiltration of CD8+ intraepithelial lymphocytes (IELs) into the epithelium and also influences the Th1 response. IELs target enterocytes for killing via apoptosis, causing destruction of the epithelial layer, and villous flattening. The combination of enterocyte apoptosis and tight junction disruption renders the epithelium more permeable, thereby facilitating access of gluten and propagation of the disease. In the continued presence of dietary gluten, chronic inflammation persists. TCR, T cell receptor.

supporting the idea that a shared group of genes contribute to a spectrum of immune diseases [57]. In the present study, we aimed at investigating the prevalence of HLA-DQ2/DQ8 in RPL women, outside of CD. To this end, we compared RPL with healthy control women with previous uncomplicated pregnancies. Compared to controls, we found a significant increased prevalence of HLA-DQ2/DQ8 positivity in RPL group (52.6% vs 23.6%), with a 3.6 times higher odds of DQ2/DQ8 positivity. To date, we are not able to explain such preliminary observation. We suggest that HLA-DQ2/DQ8 haplotype positivity as a genetically prevalent condition in RPL women, which might be associated to an increased risk of immune disturbances. Whether a similar mechanism to that of CD can be linked to this obstetric complication needs to be investigated. We are aware that this model might appear a simplification of all the complex mechanisms underlying RPL; however, our results draw attention to a statistically significant association between HLA-DQ2/DQ8 and ANA positivity in RPL women. We previously reported a significantly higher prevalence of ANA positivity in RPL women compared to control (~50% vs 8.3%–27%). ANA are a group of autoantibodies found both in the serum of patients with autoimmune and rheumatic diseases and in the general population. As serological markers, ANA show diagnostic and prognostic significance, while their clinical utility in normal individuals is still unclear (prevalence from 3.8% to 12.8%): while many serologically positive individuals will never develop an autoimmune disease, others may be in a pre-autoimmune state [61].

In conclusion, we assessed the frequencies of haplotypes HLA-DQ2/DQ8 in RPL and control women. Our findings show for the first time that the HLA-DQ2/DQ8 alleles by themselves, outside of CD, are found more frequently in RPL women. The pathogenic, diagnostic, and therapeutical implications of such preliminary results need to be investigated. We may suggest as a possible pathogenic link that HLA-DQ2/DQ8 positivity, in presence of exogenous still unknown stimuli, may favor an immune condition with detrimental effects during early stages of pregnancy. Further studies based on larger number of women are needed to confirm our observations.

Take home messages

- It is now well accepted the significant association between recurrent pregnancy loss women (RPL) and celiac disease (CD).
- CD shows a complex non-Mendelian pattern of inheritance, involving major histocompatibility complex (MHC) genes. The common haplotypes DQ2.5, DQ2.2, and DQ8 have been shown to increase CD risk by six -fold on average.
- Approximately 25% of the general Caucasian population is HLA-DQ2/DQ8. From these genetically susceptible individuals, only 4% develop CD.
- Our observations show for the first time a higher proportion of individuals HLA DQ2/DQ8 positive in women with RPL as compared to controls (52.6% vs 23.6%; p 0.01), in the absence of diagnosis of CD.
- To date, we are not able to explain such preliminary observation. We suggest that HLA-DQ2/DQ8 haplotype positivity as a genetically prevalent condition in RPL women, which might be associated to an increased risk of immune disturbances. Whether a similar mechanism to that of CD can be linked to this obstetric complication needs to be investigated.
- We may suggest as a possible pathogenic link that HLA-DQ2/DQ8 positivity, in presence of exogenous still unknown stimuli, may favour an immune condition with detrimental effects during early stages of pregnancy. Further studies based on larger number of women are needed to confirm our observations.

Conflict of interest

Non conflict of interest to declare.

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