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HLA-DRB1 polymorphism in recurrent pregnancy loss: New evidence for an association to HLA-DRB1*07☆

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ABSTRACT

Many cases of recurrent pregnancy loss (RPL) defined as ≥3 consecutive pregnancy losses are suggested to be caused by an aberrant maternal immune response against the fetus or trophoblast. Human leukocyte antigen (HLA)–DRB1 and -DQB1 polymorphisms are associated with most autoimmune disorders and studies of HLA-DBB1 polymorphism in RPL patients are thus relevant. In previous studies, the HLA-DRB1*03 allele was found with increased prevalence in RPL patients.

We wanted to clarify whether HLA-DRB1 alleles indeed were associated with RPL among women of Caucasian descent. A total of 1078 women with unexplained RPL and 2066 bone marrow donors were HLA-DRB1-typed and subsets were also HLA-DQB1 typed.

All patients were initially HLA-DRB1-typed by DNA-based low-resolution techniques and subsets of patients and all controls were typed by high-resolution techniques.

Among patients, the HLA-DRB1*07 allele frequency was significantly increased compared with controls; OR 1.29 (95 % CI 1.09−1.52), $p < 0.0025$; after correction for multiple comparisons $pc = 0.031$. The HLA-DRB1*07/*07 genotype was highly increased in patients with RPL compared with controls: OR 2.27 (1.31−3.93), $p = 0.0027$. The frequency of the HLA-DRB1*07 phenotype in RPL patients had increased significantly ($p = 0.002$) in three studies from our group published 1994–2021. The allele frequency of HLA-DRB1*03 was not increased in RPL patients compared with controls; OR 0.96 (0.83−1.12).

In conclusion, the previous association between HLA-DRB1*03 and RPL could not be confirmed in our study whereas an association to HLA-DRB1*07 was detected for the first time. Since the latter association is a new finding, it should be confirmed in future studies.

1. Introduction

The HLA region comprises several genetic loci located on chromosome 6p21.31 and it contains the most polymorphic genes known in humans. Genes in most HLA loci are involved in interactions in the immune system through various mechanisms. So called classical HLA class I genes (HLA-A, -B and C) play important roles in transplantation immunology. In addition, class I HLA molecules seem to play a role in immune interactions of importance to pregnancy. Fetal HLA-C can interact with maternal natural killer (NK) cell receptors that may

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influence the risk of pregnancy loss (Hiby et al., 2008). The non-classical HLA-class Ib gene HLA-G probably plays an important role in human reproduction since it is strongly expressed on extravillous trophoblast, which is in close contact with the maternal immune system and almost devoid of other HLA molecules. Polymorphisms in the HLA-G gene that may affect gene expression seem to play a role in several pregnancy complications such as recurrent pregnancy loss (RPL) (Hviid et al., 2002; Wang et al., 2013; Fan et al., 2014) and low birthweight (Emmery et al., 2017).

The clinically most important HLA class II loci are HLA-DRB1, -DQA1 and -DQB1. Genes in these loci code for glycoprotein molecules constitutively expressed on the surface of B-lymphocytes and dendritic cells. Class II molecules on the surface of these cells present peptides derived from exogenous proteins (e.g. bacteria) to receptors on T-helper cells. The T cells then initiate a cellular and humoral immune response against the peptide.

The two sets of HLA genes carried by every individual are strong determinants of the repertoire of proteins against which the individual can mount immunological responses. The repertoire of autologous HLA molecules is also important for the development and specificity of a subpopulation of T regulatory (Treg) cells, which can suppress autoreactive T cells that have escaped negative selection in the thymus. Treg populations and thus autologous major histocompatibility (MHC) polymorphism seems to be important for immunological tolerance to pregnancy both in mice (Aluvihare et al., 2004; Darrasse-Jeze et al., 2006) and humans (Jin et al., 2009; Kwiatek et al., 2015).

There are many indications that RPL defined as $\geq$ 3 consecutive pregnancy losses (miscarriages and biochemical pregnancy losses) before gestational week 20 (Jauniaux et al., 2006) is associated with aberrant immune function and in particular breakage of immunological autotolerance and autoimmunity. Several autoantibodies such as antiphospholipid, antinuclear and thyroid peroxidase (TPO) antibodies can be detected with increased prevalence in these women (Christiansen et al., 1998; Ticconi et al., 2010; van den Boogaard et al., 2011) and are associated with prognosis (Bliddal et al., 2019). In addition, most clinical autoimmune diseases are more common in RPL patients than in the background population (Christiansen et al., 2008). In follow-up studies, atherosclerotic diseases with a possible background in an increased inflammatory state develop more often than expected in patients with a diagnosis of RPL or late pregnancy loss (Ranthe et al., 2013; Kessous et al., 2014; Egerup et al., 2020; Westergaard et al., 2020). Most disorders associated with autoimmunity are associated with HLA class II polymorphisms (Viatte et al., 2015) and therefore it is of interest to investigate the role of HLA class II in women with RPL.

In the largest case-control study so far of 588 Caucasian RPL patients and 562 Caucasian controls, the HLA-DRB1*03 allele was found with significantly increased prevalence in patients also after correction for multiple comparisons (Kruse et al., 2004). The association to HLA-DRB1*03 was stronger in patients with $\geq$ 4 previous pregnancy losses and in patients with RPL after a birth (secondary RPL).

In prospective studies we have reported that HLA class II alleles associated with immunity against male specific minor histocompatibility (HY) antigens, primarily HLA-DRB1*15, -DRB1*07 and -DQB1*0501/0502 exhibited a negative prognostic impact in patients with RPL after the birth of a boy (Nielsen et al., 2009; Kolte et al., 2016). Furthermore, maternal carriage of HLA-DRB1*03 and HLA-DRB1*01 (the latter in strong positive linkage disequilibrium with HLA-DQB1*0501/*0502) were previously reported to be associated with a significantly reduced pregnancy prognosis in all RPL patients (Christiansen et al., 1993). The topic of HLA-DRB1 polymorphisms and RPL is controversial: in a systematic review and meta-analysis of 8 case-control studies, HLA-DRB1*04 and HLA-DRB1*15 were found to confer susceptibility to RPL whereas HLA-DRB1*03 was only borderline associated (Meuleman et al., 2015). However, only three of the studies were performed in Caucasians. In the study of Meuleman et al. (2015) a meta-analysis of studies of HLA sharing (also called HLA matching) was also performed. In matching studies, instead of comparing the frequency of HLA alleles in women with RPL with that in controls, the frequency of sharing 0, 1 or 2 alleles in different loci between women with RPL and their husbands (as a proxy for feto-maternal HLA sharing) is compared with the sharing rate in control couples. In the meta-analysis of Meuleman et al. (2015) significantly increased sharing of HLA-B and HLA-DR alleles was found in RPL couples. Increased HLA allele matching between women with RPL and their fetuses have been suggested to result in decreased immunological allorecognition of paternal antigens in the fetus that may increase the miscarriage risk.

In order to clarify the question whether particular HLA-DRB1 alleles are associated with RPL we undertook the present study, which is substantially larger than previous studies on the topic. We tested the hypothesis that HLA-DRB1*03 is significantly associated with RPL, and, in addition we investigated if there are other HLA-DRB1 alleles predisposing to or protecting against RPL.

2. Materials and methods

2.1. Patients

Between 2003 and 2016, 1778 women were referred to the national RPL clinic in Copenhagen from all over Denmark. In principle, patients needed to fulfill the criteria of three or more consecutive pregnancy losses. At referral, comprehensive information about the patients' reproductive history, previous investigations and treatments, menstrual cycle and ancestry (country of origin) were entered into a database (Danish Data Protection Agency approval numbers 2009-41-3686/2012-58-0004). Blood samples were routinely taken from all patients for HLA-DRB1 typing by low-resolution and excess DNA storage. High-resolution HLA-DRB1 typing and HLA-DQA1 and -DQB1 typing was undertaken in HLA-DRB1*07 positive patients.

In this study, we excluded a total of 700 patients due to various causes (Fig. 1). We aimed to only include women of Caucasian ethnicity. Women with origin from Asia (except Russia) and Africa and women of Afro-American ancestry from Europe and North and South America were excluded. Menstrual disturbances were defined as the majority of cycles $<$21 or $>$35 days within the last year before referral. Additional exclusion criteria were significant chromosomal abnormalities (primarily balanced translocations) in any of the parents and significant uterine malformations (septum or bicornuate uterus) or fibromas detected at hysteroscopy or hysterosalpingography. Women with exclusively biochemical pregnancies were also excluded since we believe that a substantial part of these women had experienced recurrent spontaneously resorbed ectopic pregnancies rather than recurrent intrauterine pregnancy losses (Kolte et al., 2014). A total of 1078 patients ended up being eligible for the study and had been HLA-DRB1 typed or had DNA stored for typing.

The mean age of the women was 34.08 years (SD 4.93; range 21–45) and the mean number of pregnancy losses among all the included women was 4.06 (SD 1.38; range 3–12).

Patients with autoantibodies and heritable thrombophilia factors were not excluded. There is an association between particular HLA-DRB1 alleles and autoantibody production (e.g. anticardiolipin and HLA-DRB1*03; Christiansen et al., 1998) and one of the causal links between HLA and autoimmune disease may be autoantibody production associated with the susceptibility HLA alleles. Excluding autoantibody positive patients will probably confound the results.

The only autoantibodies that have been consistently tested in our patients referred since 2011 are the antiphospholipid antibodies lupus anticoagulant and anticardiolipin and TPO antibodies. The frequency of positivity for TPO antibodies ($>$ 60 kIU/l) in 825 consecutive patients was 16.8 % and the frequency of the antiphospholipid syndrome in 511 patients who became pregnant 2011−17 was 3.5 % (Bliddal et al., 2018). In the period 2003–2007, 1.2 % of our patients had a diagnosis of systemic lupus erythematosus, 4.5 % had hyper- or hypothyreosis, 1.2 %

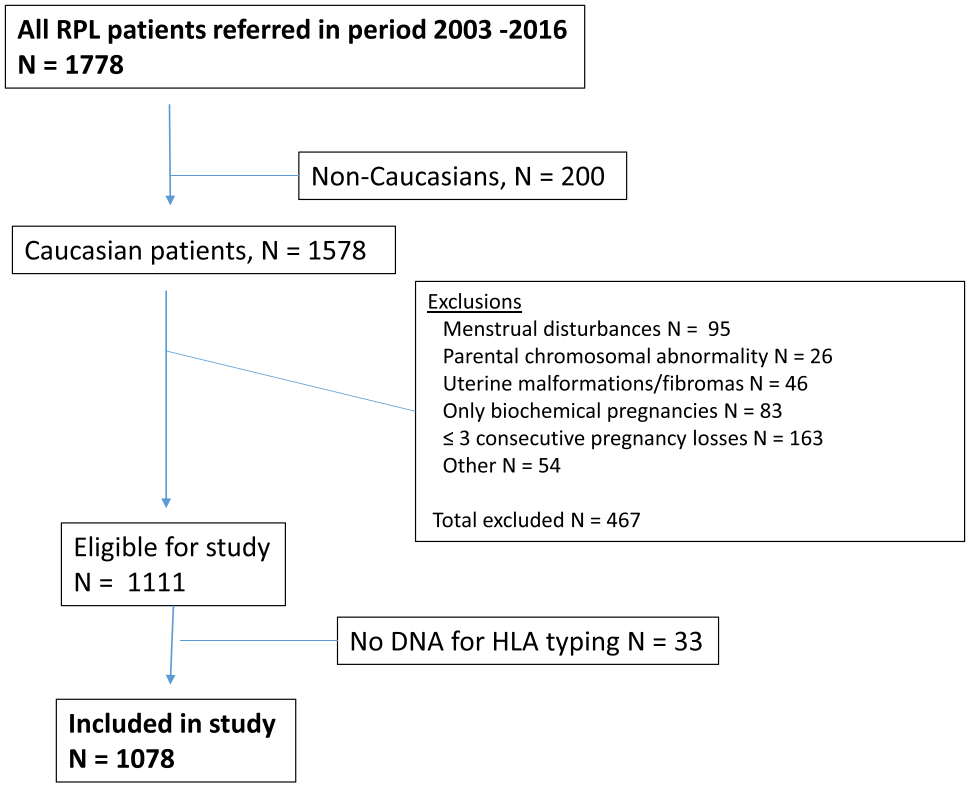

Fig. 1. Flow-chart showing causes of exclusion and numbers of excluded recurrent pregnancy loss patients in the study.

had type 1 diabetes mellitus and 1.5 % had inflammatory bowel disease (Christiansen et al., 2008).

With regard to thrombophilia factors such as the factor II and V gene variants, Protein S and C deficiency and hyperhomocysteinaemia, the ESHRE Guideline Recurrent Pregnancy Loss (ESHRE Guideline Group on RPL, 2018) suggests not to screen for hereditary thrombophilia in RPL patients since there is insufficient evidence that these factors cause RPL and no proven treatment exists. Until more is known about the impact of hereditary thrombophilia in RPL it would be wrong to select patients based on positivity or negativity for these factors to studies of other potential risk factors in RPL.

2.2. Controls

The control group comprised 2066 healthy individuals from the Danish registry of bone marrow donors (BMD), who had been HLA class II typed with high resolution at 4-digit allelic level when they volunteered for the registry. The criterion for being included in this registry was absence of known disease. The only information available regarding this group was surname, sex and age. After checking names, one individual with an Indian name was excluded whereas the other names suggested Danish ancestry. Of the controls, 56.34 % were women and their mean age was 36.74 year, (SD 9.20, range 18–64).

Since the control persons were anonymous and no extra blood samples were taken or extra analyses were performed, no consent is needed for including the controls according to the Standards of Transfusion Medicine published by the Danish Society of Clinical Immunology (Transfusionsmedicinske Standarder. Version 5.1, 2020).

2.3. HLA-typing methodology

2.3.1. DNA preparation

DNA from EDTA-treated peripheral blood was extracted either using a salting-out method as previously described (Miller et al., 1988) or using the Maxwell 16 Blood DNA kit on the Maxwell 16 Instrument. DNA from buccal swabs were extracted using Maxwell 16 Buccal Swab LEV DNA Purification Kit on the Maxwell 16 Instrument (Promega, Madison, WI, USA).

2.3.2. HLA typing with low-resolution

HLA-DRB1 typing was performed using the Luminex xMAP system LABType SSO, a reverse SSO DNA typing system (One Lambda Inc., Canoga Park, CA, USA) according to the manufacturer's instructions.

2.3.3. HLA-DRB1 and DQB1 typing with high resolution

Next generation sequencing (NGS)-based HLA typing using the Illumina MiSeq platform was performed at Histogenetics (NY, USA). HLA typing with 2x high resolution at 4-digit allelic level without ambiguities for HLA-DRB1 and HLA-DQB1 was used for large-scale HLA class II genotyping. Exon 2 and 3 were sequenced for HLA class II alleles.

2.3.4. Allele frequency versus phenotype frequencies

In the primary analysis we report the allele frequencies, which is the frequency of a specific HLA allele among all alleles in the locus; therefore, the number in the denominator is 2 x the number of individuals. The allele frequency is the most commonly used unit in HLA studies since it does not implicate a dominant mode of action of the disease susceptibility alleles and with modern techniques both alleles in the loci can be unambiguously determined.

"Phenotype frequency" is the frequency of individuals carrying a specific allele. We primarily present phenotype frequencies when our results are analyzed in a context with those of previous publications where phenotype frequencies were the unit of measure.

2.4. Statistics

All data were entered in an Access database and calculations were primarily done in Excel. Allele and phenotype frequencies were compared with $\chi 2$ test or Fisher's exact test where appropriate due to small numbers. For each table of allele comparisons, p-values were

corrected by the Bonferroni method by multiplication with the number of alleles tested except for HLA-DRB1*03, which was *a-priori* considered a susceptibility allele for RPL. ORs and their 95 % CIs were calculated for each comparison of allele or genotype frequencies. Trends in change in ≥3 frequencies were statistically evaluated by Mantel-Haenzel $\chi 2$ test for trend. Population attributable risks (PAR) were calculated by the formula: PAR = Pe (ORe – 1)/1+ Pe (ORe -1), where Pe is the prevalence of the exposure in the background population and ORe is the risk of the disease in exposed individuals. A p-value < 0.05 was considered significant.

3. Results

3.1. Allele frequencies in patients and controls

The allele frequency of HLA-DRB1*03 was not increased in RPL patients compared with the control group, OR 0.96 (95 % CI 0.83−1.12) (Table 1).

The HLA-DRB1*07 allele frequency was significantly increased compared with controls; OR 1.29 (95 % CI 1.09−1.52), $p < 0.0025$; after adjusting for multiple comparisons pc = 0.031.

In controls, we found no significant differences in the allele frequencies between the two sexes (HLA-DRB1*03: 13.6 % and 14.1 % in females and males, respectively and HLA-DRB1*07: 10.3 % and 9.1 % in females and males, respectively. ORs for RPL for the two alleles remain almost the same (HLA-DRB1*03: OR 1.00; 95 % CI 0.84–1.19 and HLA-DRB1*07: OR 1.22; 95 % CI 1.01−1,47), $p < 0.05$) if comparisons were done to only female controls.

The frequencies of all other broad HLA-DRB1 alleles were strikingly similar between RPL patients and controls (Table 1).

3.2. Phenotype frequencies in subsets of patients

The frequency of the HLA-DRB1*03 phenotype was not different between patients with primary or secondary RPL but tended to increase (p = 0.09) with the number of previous pregnancy losses (Table 2). The frequency of the HLA-DRB1*07 phenotype did not differ significantly between RPL patients with primary and secondary RPL or 3 or ≥ 4 previous pregnancy losses (Table 2). The PAR for RPL associated with HLA-DRB1*07 was 0.044.

Table 1
Frequencies of HLA-DRB1 alleles in recurrent pregnancy loss (RPL) patients and bone marrow donor controls.

HLA-DRB1*	RPL (n = 2156)	controls (n = 4132)	OR (95 % CI)	P; Pc
01	10.76	11.08	0.97 (0.82−1.14)	0.7
03	13.08	13.53	0.96 (0.83−1.12)	0.6
04	17.16	16.36	1.06 (0.92−1.22)	0.4
07	12.29	9.80	1.29 (1.09−1.52)	< 0.0025; 0.03
08	3.15	3.75	0.84 (0.63−1.12)	0.2
09	1.07	0.85	1.26 (0.74−2.14)	0.4
10	0.70	0.56	1.25 (0.65−2.40)	0.5
11	7.42	6.97	1.07 (0.88−1.31)	0.5
12	2.46	2.54	0.97 (0.69−1.35)	0.8
13	12.76	13.75	0.92 (0.79−1.07)	0.3
14	2.27	2.23	1.02 (0.72−1.45)	0.9
15	16.14	17.52	0.91 (0.79−1.40)	0.2
16	0.83	1.06	0.78 (0.45−1.36)	0.4

Pc: p-values adjusted for number of comparisons (n = 13).

Table 2
Frequencies of selected HLA-DRB1 phenotypes in subsets of recurrent pregnancy loss (RPL) patients.

HLA class II allele	Primary RPL (n = 541)[a]	Secondary RPL (n = 512)[a]	3 pregnancy losses (n = 479)	≥ 4 pregnancy losses (n = 599)
DRB1*03	23.4	24.6	22.1[b]	26.6[b]
DRB1*07	22.7	21.1	22.5	21.6

[a] Some patients could not be classified in either subgroup.
[b] p = 0.09.

3.3. HLA-DRB1*07 haplotypes in patients and controls

Table 3 depicts the distribution of the two different HLA-DRB1-DQB1 haplotypes carrying HLA-DRB1*07 alleles in RPL patients and the control group. Differences in distribution of the HLA-DRB1*07-DQB1*02 and DRB1*07-DQB1*03 haplotypes between patients and controls was not statistically significant.

3.4. HLA-DRB1 genotypes in patients and controls

In Table 4 are shown the frequencies in RPL patients and the control group of three HLA-DRB1 genotypes including the two alleles exhibiting significantly different frequencies in patients and controls in our previous and present studies. Homozygosity for HLA-DRB1*07 was found significantly more often in RPL patients compared with controls (OR = 2.27; 95 % CI 1.31−3.93, p = 0.0027). The other genotypes did not differ significantly between patients and controls.

3.5. HLA-DR3 and HLA-DR7 and RPL in historical context

Table 5 shows data from two previous publications from the Danish RPL clinics in addition to data from the present study on the frequency of RPL patients and controls being positive for the two HLA-DRB1 alleles of particular interest in this study: HLA-DRB1*03 and -DRB1*07. The frequency of the HLA-DRB1*07 phenotype in RPL patients has increased significantly from 1994 to 2021 (p = 0.002) whereas the change in the HLA-DRB1*03 phenotype frequency in patients was not significant (p = 0.20).

The frequency of the HLA-DRB1*03 phenotype has exhibited considerable variability in controls during the years (Table 5). In contrast, the frequency of the HLA-DRB1*07 phenotype has stayed stable over the years in the control populations.

3.6. Non-visualized pregnancy losses in HLA-DRB1*03 and -DRB1*07 homozygous patients

When reviewing the reproductive history in HLA-DRB1*03 homozygous patients, 29 (44.6 %) of 65 pregnancy losses could be classified as non-visualized pregnancy losses (biochemical pregnancies and

Table 3
Distribution of HLA-DRB1*07 positive haplotypes in recurrent pregnancy loss (RPL) patients and blood marrow donor controls.

DRB1-DQB1 haplotype	RPL patients (n = 221)	Controls (n = 405)	P
DRB1*07-DQA1*02-DQB1*02	71.0 %	65.4 %	0.15
DRB1*07-DQA1*03-DQB1*03	29.0 %	34.6 %	

Table 4
Frequencies of genotypes including HLA-DRB1*03 and -DRB1*07 in recurrent pregnancy loss (RPL) patients and blood marrow donor controls.

DRB1 genotype	% RPL (n = 1078)	% Controls (n = 2066)	OR (95 % CI)	P
03/03	1.58	2.18	0.72 (0.41−1.26)	0.25
07/07	2.60	1.16	2.27 (1.31−3.93)	0.0027
03/07	2.88	2.18	1.33 (0.84−2.11)	0.23

Table 5
Frequency of selected HLA-DR phenotypes in recurrent pregnancy loss (RPL) patients and fertile controls and blood or bone marrow donors in three Danish case-control studies.

	HLA-DR3 %	HLA-DR7 %
RPL 1994[a] (n = 234)	28.2[d]	14.1[e]
RPL 2004[b] (n = 354)	26.6[d]	16.9[e]
RPL 2021[c] (n = 1078)	24.6[d]	22.0[e]
Fertile controls 2004[b] (n = 202)	20.8	17.8
Donor controls 1994[a] (n = 360)	21.1	17.5
Donor controls 2021[c] (n = 2066)	24.9	18.4

[a] Christiansen et al. (1994).
[b] Kruse et al. (2004).
[c] This study.
[d] p = 0.20.
[e] p = 0.0022.

pregnancies of unknown location = PULs). In HLA-DRB1*07 homozygous patients, 35 out of 103 (34.0 %) of the pregnancy losses were non-visualized losses.

4. Discussion

The present study is by far the largest case-control study of HLA-DRB1 prevalence in patients with RPL and relevant controls and all HLA typings were done with up-to-date DNA based techniques, which will detect all broad HLA-DRB1 alleles in an individual in contrast to older techniques based on serology.

Surprisingly, when compared with the control group there was no difference in the HLA-DRB1*03 frequency in RPL patients.

In studies of genetic biomarkers in patients with a specific disease and controls, it is a matter of controversy, which is the optimal control group: individuals without the disease (e.g. a group of women with children and no pregnancy losses) or individuals selected randomly from the population? The control group of bone marrow donors was selected due to absence of disease (including autoimmune diseases), which may affect the HLA-DRB1 allele distribution in an unpredictable way.

A surprising finding in this study was that HLA-DRB1*07 was increased in the RPL group compared with controls, and this result was highly significant ($p < 0.0025$). In our previous case-control studies there was no indication that this allele confers susceptibility to RPL since OR for RPL was 0.9 in both studies (Christiansen et al., 1994; Kruse et al., 2004). However, the low p-value, which survives Bonferroni correction strengthens the belief that the association is real.

The question is why HLA-DRB1*07 has not been associated with RPL in previous studies? It is possible that previous estimates have been imprecise due to smaller numbers. However, Table 5 shows that the phenotype frequencies of HLA-DRB1*07 in RPL patients have increased significantly ($p = 0.002$) and stepwise from 14.1% to 22.0% in our three studies published from 1994 until now. In comparison, the corresponding frequencies of HLA-DRB1*03 have remained stable over the years in both the control groups of fertile women and blood and bone-marrow donors. Although the technique in our laboratory for DNA-based detection of HLA-DR polymorphisms has changed from the restriction fragment length polymorphism method (Erlich et al., 1986) to a number of polymerase chain reaction (PCR) methods (Fernandez-Vina et al., 1991), oligonucleotide probe hybridization (Saiki et al., 1986) and direct sequencing of amplification products (Gyllensten and Erlich, 1988), the stable HLA-DRB1*07 frequencies in all control groups suggest that changed techniques are not responsible for the increased DRB1*07 frequency in patients.

The increase of DRB1*07 frequency in RPL over the past 30 years may instead be explained by changes in the constitution of the patient population and its exposure to risk factors for RPL. Compared with the period before 2000, the patient population now comprises more patients with non-visualized pregnancy losses only detected by high-sensitive plasma hCG test, which only became generally available after 2000. However, among HLA-DRB1*07 homozygous RPL patients, the frequency of non-visualized pregnancy losses was similar to that in our total patient group previously published by Kolte et al. (2014), 34.0 % versus 37.8 %. Therefore, inclusion of non-visualized losses in the RPL diagnosis does not fully explain the increase in HLA-DRB1*07. Another factor that has changed significantly over the years is female age. The RPL patients have become older from 1994 to now. Egerup et al. (2016) reported a mean age of 32.5 years before 1994 and 34.4 years after 2008 in secondary RPL patients participating in randomized controlled trials. Increased mean age is expected to increase the frequency of patients getting the RPL diagnosis due to recurrent aneuploid conceptions and this may explain the weakened association between RPL and HLA-DRB1*03 but cannot explain the new association with HLA-DRB1*07. Other examples of changes in HLA associations with autoimmune disease over time have been reported. The frequencies of HLA-DRB1 alleles and genotypes providing susceptibility to type 1 diabetes were significantly lower in patients diagnosed between 1985 and 2002 compared with patients diagnosed in previous years (Hermann et al., 2003; Gillespie et al., 2004). An explanation could be that a temporal increase in environmental risk factors for type 1 diabetes have weakened the proportion of the cases caused by HLA-DRB1 alleles conferring susceptibility to type 1 diabetes. In RPL, it is possible that changes in vaginal/uterine microbiomas or endocrine disruptors in the environment over time have promoted the development of new epitopes on the fetal or trophoblast cells that are targets for HLA-DRB1*07 restricted T-helper cells.

It can be questioned whether the control group includes non-Caucasians since only one donor of presumed non-Caucasian origin was excluded from the control group whereas 11.2 % of the patients were excluded due to non-Caucasian ethnicity. There is no official registration of the ethnic origin of Danish blood donors, but in the newsletter of the organization of Danish blood donors (Bloddonor no. 119, autumn 2016) it is estimated that only 2% of Danish blood donors are of non-Danish ancestry and the frequency of non-Caucasian blood and bone-marrow donors is probably substantially lower. The vast majority of citizens in Denmark of non-Caucasian ancestry comes from the Middle East and Turkey. In these counties the HLA-DRB1*15 allele is relatively rare comprising 3.6–9.0 % in Eastern Mediterranean Arabs (Hajjej et al., 2018) and 7.5–8.0 % in Turks (Patiroglu and Akar, 2016; Esendagli et al., 2018). The HLA-DRB1*15 allele frequency of 17.52 % in our control group is very similar to the corresponding frequency of 16.72 % among 4514 Caucasian bone-marrow donors in Norway (Lande et al., 2018) suggesting a very low admixture of individuals with Arab and Turkish ancestry in our control group. The distribution of other HLA-DRB1 alleles between our controls and the Norwegian donor group is also very similar.

If both HLA-DRB1*03 (previous studies) and HLA-DRB1*07 (present study) indeed are associated with RPL, which is a situation very similar to celiac disease (Spurkland et al., 1997; Donat et al., 2009) the question is whether similar disease mechanisms lay behind the two conditions. In Caucasians, HLA-DRB1*03 is usually found on the very conserved HLA-DRB1*03-DQA1*05-DQB1*02 haplotype. In contrast, HLA-DRB1*07 is found on either the HLA-DRB1*07-DQA1*03-DQB1*03 or the

HLA-DRB1*07-DQA1*02-DQB1*02 haplotype. In celiac disease, it was found that carriers of the HLA-DRB1*03-DQA1*05-DQB1*02 haplotype or the HLA-DRB1*11(12)-DQA1*05-DQB1*03/DRB1*07-DQA1*02-DQB1*02 genotype had the highest risk of disease because these haplotypes/genotypes could form a HLA-DQ α1*05, β1*02 heterodimeric molecule which has been documented to present gliadin-derived peptides with high affinity to T-cells, which can result in celiac disease.

In Table 3 we investigated whether the HLA-DRB1*07-DQB1*02 haplotypes were over-represented among the HLA-DRB1*07 positive haplotypes in RPL patients compared with the control group; however, this was not the case. We therefore conclude that the molecular background for the possible association between HLA-DRB1*03 and HLA-DRB1*07 and RPL is not similar to that found in celiac disease: formation of a heterodimer between specific DQ α1 and β1 chains.

The OR for RPL in carriers of the DRB1*07 allele is modest: 1.29 and is so far of limited value when advising patients about causes and risk for RPL. The clinical impact must be clarified in future prospective studies. However, previous studies have provided some preliminary documentation that HLA-DRB1*07 affects the pregnancy prognosis negatively in women with secondary RPL after a boy (Kolte et al., 2016) but larger prospective studies are needed.

The PAR of the HLA-DRB1*07 allele was 0.044. This can be interpreted as if this HLA-DRB1 allele disappeared from the population 4.4 % of all RPL cases would disappear. This fraction of the RPL cases that can possibly be explained by HLA-DRB1 related factors should not be ignored.

The HLA-DRB1*07/*07 genotype was highly significantly increased (OR 2.27) in RPL patients compared with controls. If the higher genotype OR (relative to the phenotype OR of 1.29) for RPL is confirmed in a new study, this points towards a synergistic or additive effect of the two alleles carried by a woman, which can provide clues about the pathophysiological mechanisms.

What can be the pathophysiologic link between the HLA-DRB1*07 allele and RPL? It has been reported that HLA-DRB1*07 can restrict presentation of specific male-specific HY antigens to T-lymphocytes (Eljaafari et al., 2013) in vitro and it seems that women with secondary RPL, who delivered a boy before the series of pregnancy losses, the pregnancy prognosis is reduced if they are carriers of HLA-DRB1*07 (Kolte et al., 2016). HY proteins are strongly expressed on the trophoblast, which can thus be a target for harmful maternal immune reactions in HLA-DRB1*07 positive mothers. In the present study we did not find the HLA-DRB1*07 with higher frequency in secondary than in primary RPL (Table 2), which is an argument against an anti-HY pathogenesis.

In conclusion, in this large case-control study we have found strong evidence that HLA-DRB1*07 confers susceptibility to RPL in Caucasians. The study should be repeated in a new group of patients. In addition, prospective studies should clarify whether carriage of HLA-DRB1*07 impacts future pregnancy outcome in women with RPL after adjustment for confounders. If a negative prognostic impact is confirmed, HLA-DRB1 testing could be a part of the RPL screening programme. Furthermore, laboratory studies should clarify, which peptides derived from trophoblast/fetal proteins can be presented by antigen-presenting cells from HLA-DRB1*07 positive individuals to T-helper lymphocytes and initiate a cellular and humoral immune response. Such knowledge could be very helpful in the development of immunomodulatory interventions.

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Declaration of Competing Interest

The authors declare no conflict of interest

References

Aluvihare, V.R., Kallikourdis, M., Betz, A.G., 2004. Regulatory T cells mediate maternal tolerance to the fetus. Nat. Immunol. 5, 266–271.
Bliddal, S., Feldt-Rasmussen, U., Rasmussen, Å.K., Kolte, A.M., Hilsted, L.M., Christiansen, O.B., Nielsen, C.H., Nielsen, H.S., 2019. Thyroid peroxidase antibodies and prospective live birth rate: a cohort study of women with recurrent pregnancy loss. Thyroid 29, 1465–1474.
Bloddonor no. 119, 2016. Newsletter of the Danish Association of Blood Donors. Autumn.
Christiansen, O.B., Mathiesen, O., Husth, M., Lauritsen, J.G., Jersild, C., Grunnet, N., 1993. Prognostic significance of DR histocompatibility antigens in Danish women with recurrent miscarriages. Hum. Reprod. 8, 1843–1847.
Christiansen, O.B., Rasmussen, K.L., Jersild, C., Grunnet, N., 1994. HLA class II alleles confer susceptibility to recurrent fetal losses in Danish women. Tissue Antigens 44, 225–233.
Christiansen, O.B., Ulcova-Gallova, Z., Mohapeloa, H., Krauz, V., 1998. Studies on associations between human leukocyte antigen class II alleles and antiphospholipid antibodies in Danish and Czech women with recurrent miscarriage. Hum. Reprod. 12, 3326–3331.
Christiansen, O.B., Steffensen, R., Nielsen, H.S., Varming, K., 2008. Multifactorial etiology of recurrent miscarriage and its scientific and clinical implications. Gynecol. Obstet. Invest. 66, 257–267.
Darrasse-Jeze, G., Klatzmann, D., Charlotte, F., Salomon, B.L., Cohen, J.L., 2006. CD4+ CD25+ regulatory/suppressor T cells prevent allogeneic fetus rejection in mice. Immunol. Letters. 102, 106–109.
Donat, E., Planelles, D., Capilla-Villanueva, A., Montoro, J.A., Palau, P., Ribes-Koninckx, C.R., 2009. Alleleic distribution and the effect of haplotype combination for HLA type II loci in the celiac disease population of the Valencian community (Spain). Tissue Antigens 73, 255–261.
Egerup, P., Kolte, A.M., Larsen, E.C., Krog, M.C., Nielsen, H.S., Christiansen, O.B., 2016. Recurrent pregnancy loss – what is the impact of consecutive versus non-consecutive losses? Hum. Reprod. 31, 2428–2434.
Egerup, P., Mikkelsen, A.P., Kolte, A.M., Westergaard, P., Rasmussen, S., Knop, F.K., Lidegaard, Ø., Nielsen, H.S., 2020. Pregnancy loss is associated with type 2 diabetes: a nationwide case-control study. Diabetologia 63, 1521–1529.
Eljaafari, A., Yuruker, O., Ferrand, C., Farre, A., Addey, C., Tartelin, M.L., Thomas, X., Tiberghien, P., Simpson, E., Rigal, D., Scott, D., 2013. Isolation of human CD4/CD8 double-positive, graft-versus-host disease-protective, minor histocompatibility antigen-specific regulatory T cells and a novel HLA-DR7 restricted HY-specific CD4 clone. J. Immunol. 190, 184–194.
Emmery, J., Christiansen, O.B., Nilsson, L.L., Dahl, M., Skovbo, P., Møller, A.M., Steffensen, R., Hviid, T.V.F., 2017. Associations between fetal HLA-G genotype and placental weight in a large cohort of pregnant women - possible implications for HLA diversity. J. Reprod. Immunol. 120, 8–14.
Erlich, H.A., Sheldon, E.L., Horn, G., 1986. HLA typing using DNA probes. Biotechnology 4, 975–981.
Esendagli, D., Ozmen, F., Koksal, D., Onder, S., Emri, S., 2018. Association of class II human leukocyte antigen (HLA) alleles with pulmonary sarcoidosis. Sarcoidosis Vasc. Diffuse Lung Dis. 35, 143–149.
ESHRE Guideline Group on RPL, Bender Atik, R., Christiansen, O.B., Elson, J., Kolte, A. M., Lewis, S., Middeldorp, S., Nelen, W., Peramo, B., Quenby, S., Vermeulen, N., Goddijn, M., 2018. Hum. Reprod. Open hoy 004.doi: 10.1093.
Fan, W., Li, S., Huang, Z., Chen, Q., 2014. Relationship between HLA-G polymorphism and susceptibility to recurrent miscarriage: a meta-analysis of non-family-based studies. J. Assist. Reprod. Genet. 31, 173–184.
Fernandez-Vina, M., Moreas, M.E., Stastny, P., 1991. DNA typing for class-II HLA antigens with allelespecific or group-specific amplification.3. Typing for 24 alleles of HLA-DP. Hum. Immunol. 30, 60–68.
Gillespie, K.M., Bain, S.C., Barnett, A.H., Bingley, P.J., Christie, M.R., Gill, G.V., Gale, E. A.M., 2004. The rising incidence of childhood type 1 diabetes and reduced contribution of high-risk HLA haplotypes. Lancet 364, 1699–1700.
Gyllensten, U., Erlich, H.A., 1988. Generation of single-stranded DNA by the polymerase chain reaction and its application to direct sequencing of the HLA-DQA locus. Proc. Natl. Acad. Sci. U. S. A. 85, 7652–7656.
Hajjej, A., Almawi, W.Y., Arnaiz-Villena, A., Hattab, L., Hmida, S., 2018. The genetic heterogeneity of Arab populations as inferred from HLA genes. PLoS One 13 e0192269.doi: 10.1371.
Hiby, S.E., Regan, L., Lo, W., Farrell, L., Carrington, M., Moffett, A., 2008. Association of maternal killer-cell immunoglobulin-like receptors and parental HLA-C genotypes with recurrent miscarriage. Hum. Reprod. 23, 972–976.
Hviid, T.V., Hylenius, S., Hoegh, A.M., Kruse, C., Christiansen, O.B., 2002. HLA-G polymorphism in couples with recurrent spontaneous abortions. Tissue Antigens 60, 122–132.
Jauniaux, E., Farquharson, R.G., Christiansen, O.B., Exalto, N., 2006. Evidence-based guidelines for the investigation and medical treatment of recurrent miscarriage. Hum. Reprod. 21, 2216–2222.
Jin, L.P., Chen, Q.Y., Zhang, T., Guo, P.F., Li, D.J., 2009. The CD4+CD25 bright regulatory T cells and CTLA-4 expression in peripheral and decidual lymphocytes are down-regulated in human miscarriage. Clin. Immunol. 133, 402–410.
Kessous, R., Shoham-Vardi, I., Pariente, G., Sergienko, R., Holcberg, G., Sheiner, E., 2014. Recurrent pregnancy loss: a risk factor for long-term maternal atherosclerotic morbidity? Am. J. Obstet. Gynecol. 211, 414 e1-11. doi: 10.1016.
Kolte, A.M., van Oppenraaij, R.H., Quenby, S., Farquharson, R.G., Stephenson, M.D., Goddijn, M., Christiansen, O.B., 2014. Non-visualized pregnancy losses are

prognostically important for unexplained recurrent miscarriage. Hum. Reprod. 29, 931–937.
Kolte, A.M., Steffensen, R., Christiansen, O.B., Nielsen, H.S., 2016. Maternal HY-restricting HLA class II alleles are associated with poor long-term outcome in recurrent pregnancy loss after a boy. Am. J. Reprod. Immunol. 76, 400–405.
Kruse, C., Steffensen, R., Varming, K., Christiansen, O.B., 2004. A study of HLA-DR and –DQ alleles in 588 patients and 562 controls confirms that HLA-DRB1*03 is associated with recurrent miscarriage. Hum. Reprod. 19, 1215–1221.
Kwiatek, M., Geca, T., Krzyzanowski, A., Malec, A., Kwasniewska, A., 2015. Peripheral dendritic cells and CD4+CD25+Foxp3+ regulatory T cells in the first trimester of normal pregnancy and in women with recurrent miscarriage. PLoS One 10, e0124747.
Lande, A., Andersen, I., Egeland, T., Lie, B.A., Viken, M.K., 2018. HLA-A, -C, -B, -DRB1, -DQB1 and -DPB1 allele and haplotype frequencies in 4514 healthy Norwegians. Hum. Immunol. 79, 527–529.
Meuleman, T., Lashley, L.E.L.O., Dekkers, O.M., van Lith, J.M.M., Claas, F.H.J., Bloemenkamp, K.W.M., 2015. HLA associations and HLA sharing in recurrent miscarriage: a systematic review and meta-analysis. Hum. Immunol. 76, 362–373.
Miller, S.A., Dykes, D.D., Polesky, H.F., 1988. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res. 16, 1215. https://doi.org/10.1093/nar/16.3.1215.
Nielsen, H.S., Steffensen, R., Varming, K., van Halteren, A.G., Spierings, E., Ryder, L.P., Goulmy, E., Christiansen, O.B., 2009. Association of HY-restricting HLA class II alleles with pregnancy outcome in patients with recurrent miscarriage subsequent to a firstborn boy. Hum. Mol. Genet. 18, 1684–1691.
Patiroglu, T., Akar, H.H., 2016. The frequency of HLA-A, HLA-B, and HLA-DRB1 alleles in patients with acute lympho-leukemia in the Turkish population: a case-control study. Turk. J. Heamatol. 33, 339–345.
Ranthe, M.F., Andersen, E.A.W., Wohlfahrt, J., Bundgaard, A., Melbye, M., Boyd, H.A., 2013. Pregnancy loss and later risk of atherosclerotic disease. Circulation 127, 1775–1782, 2013.
Saiki, R.K., Bugawan, T.L., Horn, G.T., Mullis, K.B., Erlich, H.A., 1986. Analysis of enzymatically amplified beta-globin gene and HLA-DQ alpha DNA with allele-specific oligonucleotide probes. Nature 324, 163–166.
Spurkland, A., Ingvarsson, G., Falk, E., Knutsen, I., Sollid, L.M., Thorsby, E., 1997. Dermatitis herpetiformis and celiac disease are both primarily associated to the HLA-DQ(α1*0501, β1*02) or the HLA-DQ (α1*03, β1*0302) heterodimers. Tissue Antigens 49, 29–31.
Ticconi, C., Rotondi, F., Veglia, M., Pietropolli, A., Bernardini, S., Ria, F., Caruso, A., Di Simone, N., 2010. Antinuclear antibodies in women with recurrent pregnancy loss. Am. J. Reprod. Immunol. 64, 384–392.
Transfusionsmedicinske Standarder version 5.1. Dansk Selskab for Klinisk Immunologi., 2020. Kapitel 7: Anvendelse af blod fra donorer til fastsættelse af referenceområder, som kontrolmateriale og til forskningsformål.
Van den Boogaard, E., Vissenberg, R., Land, J.A., van Wely, M., ven der Post, J.A., Goddijn, M., Bisschop, P.H., 2011. Significance of (sub)clinical thyroid dysfunction and thyroid autoimmunity before conception and in early pregnancy: a systematic review. Hum. Reprod. Update 17, 605–619.
Viatte, S., Plant, D., Han, B., Fu, B., Yarwood, A., Thomson, W., Symmons, D.P.M., Worthington, J., Young, A., Hyrich, K.L., Morgan, A.W., Wilson, A.G., Isaacs, J.J., Raychaudhuri, S., Barton, A., 2015. Association of HLA-DRB1 haplotypes with rheumatoid arthritis severity, mortality, and treatment response. JAMA 313, 1645–1656.
Wang, X., Jiang, W., Zhang, D., 2013. Association of 14-bp insertion/deletion polymorphism in couples with recurrent spontaneous abortions. Tissue Antigens 81, 108–115.
Westergaard, D., Nielsen, A.P., Mortensen, L.H., Nielsen, H.S., Brunak, S., 2020. Phenome-wide analysis of short- and long-run disease incidence following recurrent pregnancy loss using data from a 39 year period. J. Am. Heart Assoc. 9 e015069 doi 10.1161.