NLRP7 in the Spectrum of Reproductive Wastage: Rare Non-synonymous Variants Confer Genetic Susceptibility for Recurrent Reproductive Wastage

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ABSTRACT

Background: *NLRP7* mutations are responsible for recurrent molar pregnancies and associated reproductive wastage. To investigate the role of *NLRP7* in sporadic moles and other forms of reproductive wastage, we sequenced this gene in a cohort of 135 patients with at least one hydatidiform mole (HM) or three spontaneous abortions, of which 115 are new patients.

Methods/Results: We review all mutations and correlate their number, nature, and locations with the reproductive outcomes of the patients and the histopathology of their products of conception. We demonstrate the presence of *NLRP7* mutations in two patients with recurrent spontaneous abortions and show that some rare non-synonymous variants (NSVs), present in the general population, are associated with recurrent reproductive wastage. We demonstrate that these rare NSVs are associated with lower interleukin 1 beta and tumor necrosis factor secretion and have therefore functional consequences similar to those seen in cells from patients with *NLRP7* mutations. We also attempted to elucidate the cause of stillbirths observed in 13% of our patients with *NLRP7* mutations by examining available placentas of the stillborn babies and live births from patients with mutations or rare NSVs. We show the presence of a number of severe to mild placental abnormalities, all of which are known risk factors for perinatal morbidities.

Conclusions: We recommend close follow-up for patients with *NLRP7* mutations and rare NSVs to prevent the death of their rare or reduced number of babies that reach term.

Keywords: hydatidiform mole, NLRP7, reproductive wastage, spontaneous abortion, stillbirth.

Introduction

Hydatidiform mole (HM) is an aberrant human pregnancy characterized by abnormal embryonic development, hydropic degeneration of chorionic villi, and proliferation of the trophoblast. The common form of this condition occurs once in every 250 pregnancies in western societies [1]. Common moles are sporadic, usually not recurrent, and have a complex etiology involving both genetic and environmental factors. Among women with one mole, 1-6% will develop a second mole [2, 3, 4, 5, 6, 7], depending on populations and studies, and 10-25% will experience a second reproductive wastage, mostly as a spontaneous abortion (SA) [2, 4, 8, 9].

Molar pregnancies are first diagnosed based on ultrasonography and high serum levels of beta-hCG. Definitive diagnosis is made after histopathological examination of the evacuated products of conception (POCs), which allows dividing them into complete and partial moles and distinguishing them from nonmolar spontaneous abortions. At the histopathological level, complete HMs (CHMs) do not contain embryonic tissues other than the chorionic villi and have excessive trophoblast proliferation [10]. Partial HMs (PHMs) may contain other embryonic tissues (amnion, chorion, or others) but have mild and focal trophoblastic proliferation [10]. Nonmolar spontaneous abortions may contain embryonic tissues but most do not have trophoblastic proliferation. Because histopathology is a qualitative science and lacks quantitative measurements to assess the degree and extent of trophoblastic proliferation (mild, excessive, focal, occasional, etc.), there is a wide interobserver and intraobserver variability mainly in distinguishing PHMs from SAs and less frequently in distinguishing CHMs (mainly early ones) from PHMs

[11]. In addition, epidemiological studies have shown that the frequency of moles is higher in patients with recurrent spontaneous abortions than in women from the general population [12, 13]. Also, a history of recurrent spontaneous abortions is a well-known risk factor for moles [14]. Furthermore, women with recurrent moles may have CHMs, PHMs, and SAs indicating that these three histopathological entities have, at least in some cases, the same underlying etiology and are rather a continuous spectrum of the same condition.

To date, one gene, *NLRP7*, has been shown to play a causal role in recurrent HMs (RHMs) and associated reproductive wastage [15]. Mutations in this gene have been reported by various groups and in patients from several populations demonstrating that *NLRP7* is a major gene for this condition [16, 17, 18, 19, 20, 21, 22] (http://fmf.igh.cnrs.fr/ISSAID/infevers/). Patients with familial RHMs have two defective alleles and can be homozygous for the same mutation or compound heterozygous for two different mutations. To date, only seven patients with a single defective allele in *NLRP7* have been reported [16, 17]. NLRP7 (NOD-like receptor proteins, pyrin containing domain 7) is formed by three main domains, a pyrin domain, a NACHT domain, and 9 to 10 leucine rich repeats (LRR) depending on splice isoforms. NLRPs are a subfamily of proteins with roles in inflammation and apoptosis [24]. *In vitro*, NLRP7 overexpression inhibits caspase-1 dependent interleukin 1 beta (IL1B) secretion [25]. Recently, we demonstrated that peripheral blood mononuclear cells (PBMCs) from five patients with different *NLRP7* mutations secrete lower levels of IL1B and tumor necrosis factor (TNF) as compared to PBMCs from controls (Messaed et al., submitted). We showed that

despite their low cytokine secretion, the intracellular levels of mature IL1B in PBMCs of one analyzed patient is normal, comparable to that of control cells indicating a defect after IL1B processing and most likely affecting its secretion in the extracellular milieu. We suggested that the impaired cytokine secretion and consequently inflammatory response of the patients, due to their *NLRP7* mutations, make them tolerant to the growth of the aberrant molar conceptions with no embryo and the delayed rejection of these conceptions contribute to the molar phenotype.

Because of the diagnostic overlap between moles and spontaneous abortions, we widened our selection criteria for *NLRP7* sequencing and included patients with at least 1 HM (≥1 HM) or 3 SAs (≥3 SAs). Here we report *NLRP7* mutation analysis in 135 unrelated patients with a spectrum of reproductive wastage. We show that the highest frequency of *NLRP7* mutations is found in patients with ≥2 HMs and the lowest is in patients with ≥3 SAs. We demonstrate a significant association between complete moles and the presence of at least one protein truncating mutation. We show that rare NSVs in *NLRP7* confer genetic susceptibility for recurrent reproductive wastage. Finally, we show that patients with *NLRP7* mutations and rare NSVs have variable degrees of placental abnormalities associated with increased perinatal morbidities.

Patients and Methods

Patients and Controls

The study was approved by the Institutional Review Board of McGill and collaborating institutions. All patients and controls provided written consents to participate in this

study. Patients were ascertained by (i) referral to us from various collaborators for genetic testing, (ii) recruitment from the miscarriage clinic of the McGill Reproductive Center, and (iii) referral from the Quebec and Montpellier registries of trophoblastic diseases. Selection criteria were either at least one HM (≥ 1 HM) or one trophoblastic disease or the occurrence of at least 3 spontaneous abortions (≥3 SAs). For most patients, clinical information was collected using standard pro forma recapitulating complete reproductive, medical, and family histories. One patient, 428, with one *NLRP7* mutation previously reported by our group [16], had had in her last pregnancy a prematurely born baby at 28 weeks. The baby was later diagnosed with several congenital abnormalities including bilateral club foot, intraventricular hemorrhage grade II on the left side of the brain, developmental delay, mild tracheomalacia, patent ductus atresia that required several surgeries. Blood karyotype analysis, at a resolution level of 400 bands, revealed a 46,XY normal karyotype in 11 analyzed metaphases.

For mutation analysis, control DNA were from women either from the CEPH families or from women, of European descent, from families with various inherited conditions, unrelated to pregnancy losses, and with 5 to 16 children. Beside women from the CEPH families, most controls are of Canadian origin with a significant number being of French ancestry. However, their complete reproductive history and whether they had had reproductive wastage is not known.

Mutation analysis and annotation

Mutation analysis was performed as previously described [20] by PCR amplification of genomic DNA of the 11 *NLRP7* exons followed by direct sequencing in the two directions. Sequences were analyzed using DNASTAR. In the text, we use the term mutations to indicate DNA changes, leading to protein truncations or nonsynonymous changes that were not found in any of the tested controls including those of the same, or of related, ethnicities to the patients. We use the term nonsynonymous variants (NSV) to indicate coding DNA changes leading to amino acid changes that were found in controls from any ethnic group. When possible, identified mutations and variants were phased by genotyping available parents and other family members. Nucleotide numbering for mutations and variants uses cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence, NM_001127255.1.

Cytokine secretion and western blotting

Peripheral blood mononuclear cells were separated using Ficoll, counted, and cultured in the absence or the presence of ultrapure lipopolysaccharides (LPS) (1µg/ml) (Cedarlane 423[LB] from E. coli 0552:B5) for 24 hours. Supernatants were collected and assayed by ELISA for IL1B and TNF secretion (BD Biosciences). Statistical analyses were done using ANOVA single factor analysis given it is the appropriate comparison test to deal with a single independent variable between all the compared values. P-value <0.05 was considered as statistically significant. Western blot analysis was performed using monoclonal antibodies directed against human IL1B (Cell Signaling technology, USA), alpha-tubulin (Cell Signaling technology, USA) (1:1000) and beta-actin (Chemicon international). Protein bands were revealed using the Hyperfilm ECL Western blotting

detection reagents (GE Healthcare, USA) and quantified by Image J software (http://rsb.info.nih.gov/ij/).

Histopathology

For histopathological diagnoses of CHM, PHM, and SA, a total of 105 tissue sections from 31 POCs were stained with haematoxilin and eosin, examined independently by two pathologists with large expertise in early pregnancies, scored for four parameters, and classified as CHM, PHM, and SA. The four parameters are the presence of nucleated red blood cells inside chorionic villi, presence of fetal membranes or tissues beside chorionic villi, the degree of trophoblast proliferation, and the degree of hydropic changes. For late and term placentas tissues were screened independently by two other pathologists with extensive expertise in term placentas.

RESULTS

***NLRP7* mutations in the spectrum of reproductive wastage**

In this study, we sequenced *NLRP7* in 135 unrelated patients with ≥1 HM or ≥3 SAs, of which 115 are new patients. Of the 135 unrelated patients 45 had had ≥ 2 HMs, 64 had had only 1 HM (with or without other reproductive wastage), and 26 had had ≥3 SAs (fig 1). The highest frequency of mutations was found in patients with ≥2 HMs, 60% (26 out of 45 patients), followed by patients with one HM, 13% (8 out of 64 patients), then patients with ≥3 SAs, 8% (2 out of 26 patients). Among the eight patients with 1 HM and *NLRP7* mutations, six (75%) had had at least two additional reproductive wastage, one

had had one mole and two live births, and the remaining had had one mole, but no data are available about her other reproductive outcomes. This indicates that *NLRP7* mutations predispose patients for recurrent reproductive wastage rather than for sporadic moles. Among the 26 women with ≥3 SAs, two have *NLRP7* mutations. One had had 2 live births and 7 SAs and is heterozygous for R156Q. The second patient had had four SAs, one of which led to a gestational trophoblastic disease that required methotrexate treatment, and is heterozygous for A719V. Both mutations, R156Q and A719V, were previously seen in women with moles [16] and were not found, respectively, on 300 and 200 chromosomes from control women of matching ethnicity to that of the two patients (both of European descent). This demonstrates that *NLRP7* is also responsible for some cases of recurrent spontaneous abortions and that the variability in the reproductive outcomes of patients with the same mutation may be due to the genetic background of the patients, environmental factors, or both. Our data are in agreement with previous epidemiological data showing the occurrence of a second reproductive wastage in 10-25% of women with a prior HM [2, 4, 8, 9].

Eight new mutations and variants including three protein-truncating

Among 135 unrelated patients sequenced, 36 had at least one protein-truncating mutation or non synonymous variant that were not seen in controls of matching ethnicity. Among these mutations/variants, there were a total of 28 different mutations/variants, of which 20 mutations were previously reported [15, 16, 17, 18, 19, 20, 21, 22, 23] and eight are new mutations or variants. Three of the new mutations are protein-truncating,

c.930_931del, p.Gln310HisfsX38; c.1018_1020delinsCAAAA, p.Glu340GlnfsX11; c.2791_2792delTG, p.Cys931X and are most likely pathogenic. The remaining five are missense, c.750C>A, p.Phe250Leu; c.1018G>A, p.Glu340Lys; c.1169G>A, p.Arg390His; c.1237C>T, p.Arg413Trp, and c.2444G>A, p.Arg815His, and none of them was found in controls from several ethnic groups (supplementary table 1). These five missense variants could (i) be pathogenic, (ii) increase the susceptibility of the patients for reproductive wastage, or (iii) be simply very rare variants that were not seen in our cohort of controls from the general population. Future studies are needed to clarify their status with respect to reproductive wastage. We note that none of these new missense variants is reported on the 1000 Genome Database website. Using Polyphen 2, a software that predicts possible impact of an amino acid substitution on the structure and function of a protein using structural and comparative considerations, two missense mutations, Glu340Lys and Arg413Trp, were predicted by Polyphen-2 to be probably damaging while the remaining, Phe250Leu, Arg390His, and Arg815His, were predicted to be benign (table 1). The reproductive outcomes of the patients with the new mutations and variants are shown in the supplementary materials, table 2.

Genotype-Phenotype Relationships of Mutations in *NLRP7*

Patients with one NLRP7 defective allele have better reproductive outcomes

Among the 36 unrelated patients with mutations from the three groups (≥ 2 HMs, 1 HM, or ≥3 SAs), 24 had two defective alleles and 12 had a single defective allele. Comparing the reproductive outcomes of patients with one and two defective alleles (including

related patients in familial cases) revealed that patients with one defective allele had significantly more live births (18.4% versus 2.5%), more SAs (37% versus 17.4), and less HMs (34.1% versus 73.5% of their pregnancies) than patients with two defective alleles (Fisher exact test, p-value=2.809e-06) (fig 2A). Among the 12 patients, each with one defective allele, seven had had a single mole with other forms of reproductive wastage. These data indicate that patients with one identified defective allele most likely have a single defective allele and consequently better reproductive outcomes than individuals with 2 mutated alleles who have a much greater chance of having recurrent molar pregnancies.

Table 1. Correlation between predicted effects of missense mutations and variants and reproductive outcomes of the patients

Substitution	Polyphen-2 score	Our patients			General population
		Reproductive wastage	Live birth	Number of unrelated patients	
C399Y	0.999	1 HM	yes[#]	1	no
P716A	0.997	≥2 HMs	no	1	no
N913S	0.994	≥2 HMs	exceptionally[$]	5	no
G380R	0.987	1 HM	no	1	no
L398R	0.968	≥2 HMs	no	1	no
L964P	0.968	≥2 HMs	no	1	no
E340K	0.962	1 HM	no	1	no
R693W	0.959	≥2 HMs	no	3	no
D657V	0.951	≥2 HMs	no	1	no
A719V	0.942	1 HM or ≥3 SAs	no	2	no
R413W	0.915	1 HM	yes	1	no
C84Y	0.909	1 HM	no	1	no
R693P	0.906	≥2 HMs	exceptionally[$]	9	no
K277Q*	0.843	≥2 HMs	no	1	no
M192L*	0.701	≥2 HMs	no	1	yes
D722G	0.677	≥2 HMs	no	1	no
K379N	0.663	≥2 HMs	no	2	no
T1028A	0.478	≥2 HMs or 1 HM	yes	2	yes
R701C	0.419	≥2 HMs	no	1	no
F250L	0.280	1 HM	yes	1	no
R693Q	0.273	≥2 HMs	no	1	no

M427T	0.189	1 HM	yes	2	yes
L750V	0.110	≥2 HMs	no	2	no
R156Q	0.103	≥2 HMs or ≥3SAs	yes	2	no
K511R	0.087	≥3 SAs	yes	3	yes
R390H	0.0.041	1 HM	yes	1	no
F430L	0.033	1 HM	yes	2	yes
L311I	0.025	1 HM or ≥3 SAs	yes	2	yes
Q310R	0.007	1 HM or ≥3 SAs	yes	2	yes
A481T	0.007	≥2 HMs or 1 HM or ≥3 SAs	yes	22	yes
V319I	0.005	≥2 HMs or 1 HM or ≥3 SAs	yes	47	yes
V699I	0.003	1 HM	n.a.	1	yes
G487E	0.002	1 HM or ≥3 SAs	yes	19	yes
R815H	0.001	1 HM	n.a.	1	no

Polyphen-2 scores vary between 1 (for the most severe substitution) and 0 (for the most benign) and are listed by decreasing severity from top to bottom. n.a., indicates no available data about the other reproductive outcomes of the patient. Different outcomes in different patients are indicated by "or". * indicates missense variants found in patients on haplotypes carrying other mutations; # indicates the presence of several congenital malformations in the live birth and are described in the Materials and Methods'section; $ indicates a single live birth in one patient who is compound heterozygous for N913S and R693P among 6 patients with N913S (who had had a total of 24 pregnancies) and 10 patients with R693P (who had had a total of 36 pregnancies).

Protein-truncating mutations are associated with repeat CHMs

To investigate possible correlations between the nature of the mutations and the histopathological types of the molar tissues, a total of 105 tissue sections from 31 POCs from 13 patients (from 12 unrelated families) with *NLRP7* mutations were examined, scored, and diagnosed independently by two pathologists. Both pathologists noted that recurrent molar tissues from patients with *NLRP7* mutations have, in general, less trophoblastic proliferation than common sporadic moles. There was an agreement between the diagnoses of the two pathologists in 80% of the cases. Among 10 HMs from patients with at least one protein-truncating mutation, 9 were diagnosed as CHMs by the two pathologists (fig 2B). Patients with missense mutations had more variability in the

histopathological diagnosis of their POCs (fig 2B). These data demonstrate that repeat CHMs is the most severe phenotype caused by *NLRP7*.

The association between CHMs and protein-truncating mutations prompted us to compare the frequencies of protein truncating mutations in all reported familial and singleton cases of recurrent moles with the hypothesis that if protein truncating mutations were associated with the severe phenotype, we would expect them to be higher in familial than in singleton cases since their presence would have favored the manifestation of moles in all family members carrying them. A recapitulation of these cases showed a higher frequency of protein-truncating mutations in familial than in singleton cases, 52.17% versus 32.71% (supplementary table 3). Although, this association is not statistically significant, it indicates that some missense mutations are less penetrant and consequently not all family members carrying them manifest moles.

Distribution of mutations and variants in the three NLRP7 domains

The distribution of the different mutations and variants found in the three *NLRP7* domains is shown in fig 2C, 2D. In this cohort of patients, protein-truncating mutations were only found in patients with at least 2 HMs. Patients with only 1 HM or at least 3 spontaneous abortions had only missense variants (fig 2D). The distribution of all *NLRP7* mutations and variants in our patients is similar to those reported by all groups (including ours), which are listed on INFEVERS (http://fmf.igh.cnrs.fr/ISSAID/infevers/) (fig 2D). The highest number of missense mutations (62%) is observed in the LRR which represent only 36% of the total size of the protein. This data confirms the importance of the LRR

for the normal function of *NLRP7* as previously suggested [22]. This suggestion is further corroborated by the fact that only 1 NSV, V699I, in the LRR has been seen among all controls we analyzed from several ethnic groups as compared to 12 in the NACHT domain (319 aa), which is even 15% shorter than the LRR domain (369 aa) (fig 2D).

The predicted functional consequences of all missense mutations by polyphen-2 (http://genetics.bwh.harvard.edu/pph/) showed the association of missense variants with mild predicted functional consequences with the category of 1 HM or ≥ 3 SAs while severe mutations are associated with the occurrence of ≥ 2 HMs (table 1).

Rare non-synonymous variants in *NLRP7* are associated with recurrent hydatidiform moles and spontaneous abortions

To investigate whether the non-synonymous *NLRP7* variants predispose women to reproductive wastage, we removed from our cohort of 135 women those with at least one mutation and those of non-European origin and compared the frequencies of NSVs in 53 European patients with no mutations in *NLRP7* and 155 controls of European descent (table 2). This analysis revealed a statistically significant association between c.1441G>A, p.A481T and reproductive wastage (Chi^2=6.24, p-value=0.012). Among the 53 patients, 12 had had a single HM with either no data about their other reproductive outcomes or with normal pregnancies. When these cases were removed from the sample and only cases with one mole and at least another reproductive wastage or patients with ≥3SAs were included, the association with A481T was more significant (Chi^2=7.435, p-value=0.0063). Five other rare NSVs were more frequent in the patients than in controls,

but did not reach individually statistical significance (table 2). We then looked for association between the presence of any of the NSVs or any of the rare NSVs, listed in table 2, and reproductive wastage and found significant association with patients with 1 HM or ≥ 3SAs (p-value=0.0003) that was even higher after removing cases with 1 HM and no other reproductive wastage (p-value=0.000012). We note that none of the patients with two *NLRP7*defective alleles had any of the rare NSVs. Among patients with a single defective allele, three had one or several rare NSVs. Parental DNA was available in one case and allowed us to show that the rare variant is inherited on a different parental haplotype than the mutation. Altogether, our data support the role of A481T and the other rare NSVs in the genetic susceptibility for recurrent reproductive wastage.

Table 2. Frequencies of non-synonymous *NLRP7* variants in patients and controls of European descent

Variant	Minor alleles	Controls (n=105-155)	≥1 HM or ≥3 SA			≥1 HM and another RW or ≥3 SA		
			Patients (n=53)	Chi^2	p-values	Patients (n=40)	Chi^2	p-values
Q310R*	R	0.006	0.018	1.261		0.024	2.043	
L311I*	I	0.009	0.018	0.551		0.024	1.098	
V319I	I	0.185	0.169	0.725		0.183	0.002	
M427T*	T	0.009	0.009	0.987		0.012	0.038	
F430L*	L	0.004	0.009	0.237		0.012	0.469	
A481T*	T	0.064	0.132	6.24	**0.012**	0.159	**7.435**	**0.0063**
G487E*	E	0.035	0.056	0.899		0.061	1.076	
K511R*	R	0.018	0.028	0.338		0.037	0.904	
Any of the above		0.479	0.66	**4.52**	**0.033**	0.750	**8.401**	**0.0037**
Any rare NSV		0.177	0.471	**13.018**	**0.0003**	0.550	**19.198**	**0.000012**

n, indicates the number of subjects in each category. RW, indicates a reproductive wastage. A total of 155 controls were analysed for all variants, except for M427T, F430L, and K511R, for which 105 controls were analyzed. MAF, indicates minor allele frequency. Two by two contingency table was used for MAF higher than five in patients or controls, and Fisher exact test for values equal or lower than 5 (http://www.quantitativeskills.com/sisa/distributions/binomial.htm). Only significant p-values are indicated. Rare NSV indicates those with MAF ≤0.064 and are indicated by asterisks.

Low IL1B and TNF secretion by mononuclear blood cells from patients with A481T

Recently, we have shown that peripheral blood mononuclear cells (PBMCs) from five patients with *NLRP7* mutations have reduced levels of IL1B and TNF secretion despite the fact that they have normal level of intracellular mature IL1B (Messaed et al., submitted). To investigate the potential functional consequences of A481T and the other rare NSVs on IL1B and TNF secretion, PBMCs from 5 patients, 698 (p.[V319I;A481T;G487E];[=]), 754 (p.[A481T];[=]), 819 (p.[A481T];[=]), 821 (p.[Q310R(;)L311I(;)A481T]), 830 (p.[A481T];[=]), carrying A481T with and without other NSVs and 7 different controls without A481T and any of the other rare NSVs were stimulated *ex vivo* and the levels of cytokines were determined by ELISA. This analysis demonstrated that patients' cells secrete statistically lower levels of IL1B and TNF as compared to control cells (p-value<0.001) (fig 3A). We then looked at the levels of intracellular production of pro and mature IL1B by cells from these 5 patients and one control using western blot analysis. We found variable levels of intracellular pro and mature IL1B in different patients (fig 3B) similar to those reported in healthy subjects [26]. In all the analyzed patients, the intracellular levels of mature IL1B mirrored those of pro-IL1B demonstrating that NSVs in *NLRP7* do not impair IL1B cleavage. This result is in agreement with our previous observation in one patient with a mutation, G380R, in *NLRP7* (Messaed et al., submitted). We then looked at the ratios of intra and extracellular IL1B between cells from each of the five patients and the same control cultured, stimulated, and assayed at the same time. We found that the ratios of secreted IL1B by patient cells relative to control cells (patient/control) are lower than the ratios of their

intracellular mature ILIB (fig 3C). These findings are in agreement with our previous observation in a patient with a mutation in *NLRP7* and demonstrate that A481T and the other rare NSVs have functional consequences and reduce cytokine secretion upon stimulation with LPS. Altogether, these data demonstrate the association of A481T and rare NSVs with lower cytokine secretion similar to those observed in patients with *NLRP7* mutations, and support further the role of these rare NSVs in conferring genetic susceptibility for reproductive wastage.

Increased perinatal morbidities and placental abnormalities in patients with *NLRP7* mutations or rare NSVs

To date, six of our 46 patients with at least one *NLRP7* mutation (13 % of our patients) had had 7 stillbirths (3.4 % of their pregnancies). Tissues from all the placentas of these stillborn babies were not available to us for evaluation. The descriptions provided by the patients for four cases indicated the death of morphologically normal babies (supplementary table 5). Medical reports were available for two cases (supplementary table 6). In one case, the patient manifested, at 26 weeks of gestation, preeclampsia, placental abruption. Infarction and calcification were diagnosed by histophathological examination of the placenta of the delivered baby who died later. In the second case, a placental haematoma was diagnosed by ultrasonography after the intrauterine demise of the baby.

In an attempt to understand what could have caused the death of these babies, we retrieved placental tissues from one stillbirth and 8 live births from 6 patients with

NLRP7 mutations or with rare NSVs, all of whom are living in Canada. Histopathological evaluation of the placentas by two pathologists with extensive expertise in term placentas revealed a number of abnormalities (table 3). This evaluation showed that only 2 of the 9 analyzed placentas did not have any abnormality and 6 had one to several abnormalities with variable severities. These abnormalities included mild to severe chorioamnionitis, inflammation of the chorion and amnion that was seen in 3 placentas (supplementary fig 1); mild chorangiosis, a placental sign associated with prolonged hypoxia that was seen in 3 placentas; decidual necrosis that was seen in 3 placentas; and dysmature stem chorionic villi, seen in 2 placentas (supplementary fig 1). The abnormalities seem to correlate with the severity of the allele, with severe abnormalities in patient 428 who has a mutation and milder abnormalities in patients with rare NSVs.

We previously showed that patients with *NLRP7* mutations and variants have postzygotic cleavage abnormalities. To investigate whether the abnormalities observed in the placentas from of the stillborn and the malformed male babies of patient, 428, with a mutation in *NLRP7,* are caused by placental mosaicism and the presence of androgenetic cells with two X chromosomes, we used fluorescent in situ hybridization and probes for the Y and the X chromosomes. This analysis did not reveal any XX cells in five available sections from the two placentas and all cells from the two placentas had normal numbers of sex chromosomes with one X and one Y. We then searched for other chromosomal aneuploidies with probes from four autosomes and did not identify mosaicism or aneuploidies in the available tissues from the two placentas of patient 428.

Discussion

In this study, we report *NLRP7* mutation analysis in a cohort of 135 women with at least one HM or 3 SAs, of which 115 are new patients. We show genotype-phenotype correlations between the number of defective alleles, their nature, and their locations with the reproductive outcomes of the patients. We found that two defective alleles are associated with more severe reproductive outcomes than one defective allele. Also, protein truncating mutations were associated with CHM and were more frequent in familial than singleton cases of recurrent moles. Because of their severity, protein-truncating mutations are less likely to be modulated by the genetic background of the patients or environmental factors and consequently manifest as CHM in all family members carrying two protein-truncating alleles. In addition, the relatively more important trophoblastic proliferation in patients with protein-truncating mutations will favor the histopathological diagnoses of the POCs as complete moles by pathologists and consequently favor their familial manifestation. However, missense mutations were, in general, less severe and were associated with more variability in the reproductive outcomes of the patients. For instance, mutations R156Q and A719V, now found in two patients with several SAs, were previously reported in two patients with moles, 647 and 636 [16]. Among the two previously reported patients with moles, one had had one live birth and three moles, of which two are PHMs, and the second patient had had two live births, one partial mole and one spontaneous abortion, and other earlier forms of fetal losses [16]. Moreover, variants Q310R, L311I, and A481T were found in one patient with 1 CHM and 1 ectopic pregnancy while her mother and sister carrying the same

variants on the same haplotype had had only SAs (case MoSw177 supplementary fig 2). Altogether, these data confirm the overlap between the etiologies of SAs and moles and indicate that other genetic and environmental factors modulate the defect.

Of the analyzed patients, 41.9% did not have any mutation, but had one or several NSVs. Comparing the frequencies of each of the NSVs, individually or combined in our cohorts of European patients and controls showed a significantly increased association in the patients. These associations were stronger when patients with sporadic moles and no other reproductive wastage were removed. Despite the fact that we included in this analysis all the patients referred to our laboratories that fit our criteria (≥1 HM or ≥3 SAs), two factors may have biased the ascertainment of our samples, (i) our historical interest in the pathology of recurrent moles and (ii) the excellence of McGill Reproductive Center in reproductive medicine. These two factors may have both favored the ascertainment of severe cases with an underlying genetic defect. Nevertheless, our data indicate that *NLRP7* is not only a major gene for recurrent moles, but may also be associated with recurrent spontaneous abortions and a wider spectrum of reproductive wastage. Another interesting finding in this study is that the clinical entity of recurrent SAs, despite its emotional and economical burden on the patients, seems to be, at least in some cases, a milder form of the more severe molar phenotype. This provides an explanation of the lack of Mendelian inheritance of recurrent spontaneous abortions in families and consequently the lack of success in identifying genes responsible for this clinical entity.

Our cytokine data on patients with A481T with and without other rare NSVs show that these patients, regardless of their reproductive outcomes, with or without HMs, have impaired IL1B and TNF secretion similar to those seen in patients with *NLRP7* mutations. These data demonstrate that rare NSVs in *NLRP7* have functional consequences and support further their roles in conferring susceptibility for recurrent reproductive wastage.

To further investigate the functional consequences of *NLRP7* mutations and rare NSVs, we wanted to know what happens in the other term pregnancies of these patients. Histopathological examination of eight placentas from four patients revealed chorioamnionitis, chorangiosis, and necrosis, all of which reflect the known functions of *NLRP* genes, inflammation and apoptosis, and are known factors associated with gynecological morbidities and perinatal mortalities. Also, among our patients with *NLRP7* mutations, 6 had had 7 stillbirths. To investigate the causes of these stillbirths, we examined the histopathology of available placentas and looked for mosaicism and aneuploidies. The absence of identified mosaicism and aneuploides in two studied placentas does not exclude their presence in other regions of these placentas, but the apparent cause of these stillbirths is the various placental abnormalities. The most severe identified abnormality in the two placentas of patient 428 with C399Y was chorioaminionitis, which is a well-known cause of stillbirths and perinatal mortalities. This suggest that normal *NLRP7* has other roles in late gestation (22-40 weeks), probably in down regulating normal physiological, or pathogen-induced, inflammation that seem to be severely or mildly impaired by mutations and rare NSVs. Also, the impaired cytokine

secretion and consequently inflammatory response by PBMCs from patients with *NLRP7* mutations and variants may favour the ascending of lower reproductive tract microorganisms and contribute to the preterm rupture of membranes seen in some patients and the occurrence of stillbirths. Altogether, our data indicate that patients with *NLRP7* mutations and variants are at higher risk for pregnancy complications and perinatal mortalities, an understudied area of medicine affecting a large number of pregnancies. Our data are in agreement with a previous study showing an increased incidence of gynaecological morbidities and perinatal mortalities in women with recurrent spontaneous abortions [13]. In addition to their psychological and emotional burden on couples, perinatal mortalities constitute an important economical burden on health systems. Therefore, pinpointing their association with *NLRP7* mutations and variants will open new avenues of research to better dissect the exact role of *NLRP7* in these conditions and contribute to the identification of other genes and mechanisms underlying perinatal mortalities.

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Legends to Figures

Figure 1. Summary of *NLRP7* mutation analysis of 135 unrelated patients with a spectrum of reproductive wastage. NSV stands for non-synonymous variant, SA, for spontaneous abortion, HM, for hydatidiform mole.

Figure 2. (A) Comparison between the reproductive outcomes of patients with one or two defective alleles. n, indicates the total number of pregnancies of patients from each category. In this figure, only major categories of reproductive wastage are shown, the other rare forms (blighted ovum, ectopic pregnancy, elective abortion, malformed baby) observed in small numbers (between 2 and 6) were either removed or fused with related categories. (B) Correlation between the nature of *NLRP7* mutations and the histopathological diagnosis of products of conception (POCs) established by two pathologists. (C) Genomic and protein structures of NLRP7. (D) Distribution of the different types of *NLRP7* mutations in its three domains. Distribution of mutations and variants found in our three categories of patients and of mutations listed on INFEVERS (http://fmf.igh.cnrs.fr/ISSAID/infevers/). "inter", indicates amino acid between two domains; and "after", indicates amino acids after the LRR domain.

Figure 3. (A) IL1B and TNF secretion by *ex vivo* stimulated PBMCs with lipopolysaccharides (LPS) from 5 patients with A481T and other rare NSVs in *NLRP7*. The patients included in this analysis are 698, 754, 819, 821, 830. Each patient has one copy of A481T with or without other rare NSVs (Supplementary table 4 and supplementary figure 2). PBMCs from 7 subjects without A481T and any of the other rare NSVs were used as controls. Patients carrying A481T and other rare NSVs secrete significantly lower amounts of IL1B and TNF than controls not carrying these variants ($p<0.0001$). (B) Cell lysates of LPS stimulated PBMCs from five patients and the same control were subjected to immunoblots to determine the levels of pro-IL1B and mature IL1B. The levels of mature IL1B were normalized to those of β-actin. IL1B is not constitutively expressed by PBMCs. Upon stimulation, PBMCs from the patients and control produce variable amounts of intracellular pro and mature IL1B as reported in healthy subjects and none of them has defective IL1B processing (C) Comparison were performed between the ratios of patient-to-control (patient/control) of intracellular mature IL1B, quantitated by the Image J software, and the ratios of secreted IL1B in the extracellular milieu measured by ELISA (patient/control). This analysis showed that patients' cells secrete lower amounts of the produced mature intracellular IL1B than controls.

Legends to Supplementary materials

Supplementary figure 1. (A) Histopathology of the placenta from the malformed baby of patient 428 showing marked chorioamnionitis (arrows). (B) Placenta from term pregnancy of patient 754 displaying oedematous and dysmature stem villi with chorangiosis (arrows).

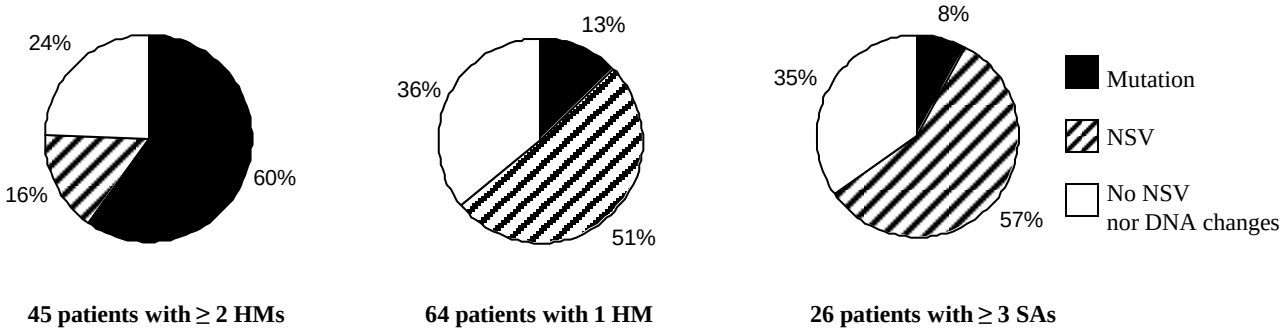
Table 3. Summary of histopathological evaluations of the placentas of patients with *NLRP7* mutations and at least one rare NSVs

Case ID	Patient ID	Mutation or Variant	N	Clinical information & Gross Morphology	Pathologist 1	Pathologist 2
MoCa57	428	p.[V319I;G487E];[V319I;C399Y]	2	GA 22 w. SB of a morphologically normal male fetus, baby weight 465g, 27 cm length, placenta 10x10 cm, 163 grams, cord inserted centrally and mesures 11 cm. No histopathology done on the baby. The patient had vaginal spotting since 10 w, high βHCG 160,625 U/L, vaginal infection, antibiotics treatment, bleeding again around 21 w, lower abdominal pain few days before the stillbirth and was admitted to the hospital. Placenta removed manually.	Immature placenta, mild chorioamnionitis, decidual necrosis.	Chorionitis, subchorionic hemorraghe
			4	GA 28 1/7 w, male live birth.True knot. Cervical incompetence, complete microbiology workout negative, cerclage, bed rest, and antibiotic treatment. Preterm labor and rupture of membranes. Chorioamnionitis. Cesarean section. Accessory lobe. The baby was later diagnosed with several congenital malformations described in Patients and Methods.	Immature placenta, marked chorioamnionitis, surface vasculitis, funisitis, deciduitis; mural thrombosis of surface vessels.	Advanced villous maturation, acute vasculitis and funisitis. Severe acute chorioamnionitis and deciduitis
MoCa209	804	p.[V319I(;) R156Q]	2	GA 40 6/7 w, 3.3kg female, 615 g,18x14x2.5, cord 30 cm, 3 vessels	Normal	Normal
			2	GA 39 3/7 w, 530g, 17x16x3 cm, cord 44 cm, 3 vessels, false knot	Normal	Normal
MoCa182	754	p.[A481T];[=]	1	Live birth of a girl	Normal placenta with minimal necrosis of decidua.	Dysmature stem villi and mild chorangiosis
MoCa207	802	p.[G487E];[=]	2	GA 41 W, 824g	Chorangiomas in the surface near the insertion of the cord	Prominent perivillous fibrin agregates, chorangiosis
MoCa210	806	p.[V319I(;) A481T]	1	GA 35 1/7 w, spontaneous vaginal delivery of a male baby, 720 g, 21x18.5x2.8, 76 cm, 1.4 cm, cord twisted, one knot, 3 vessels	Normal	Chorangiotic changes identified

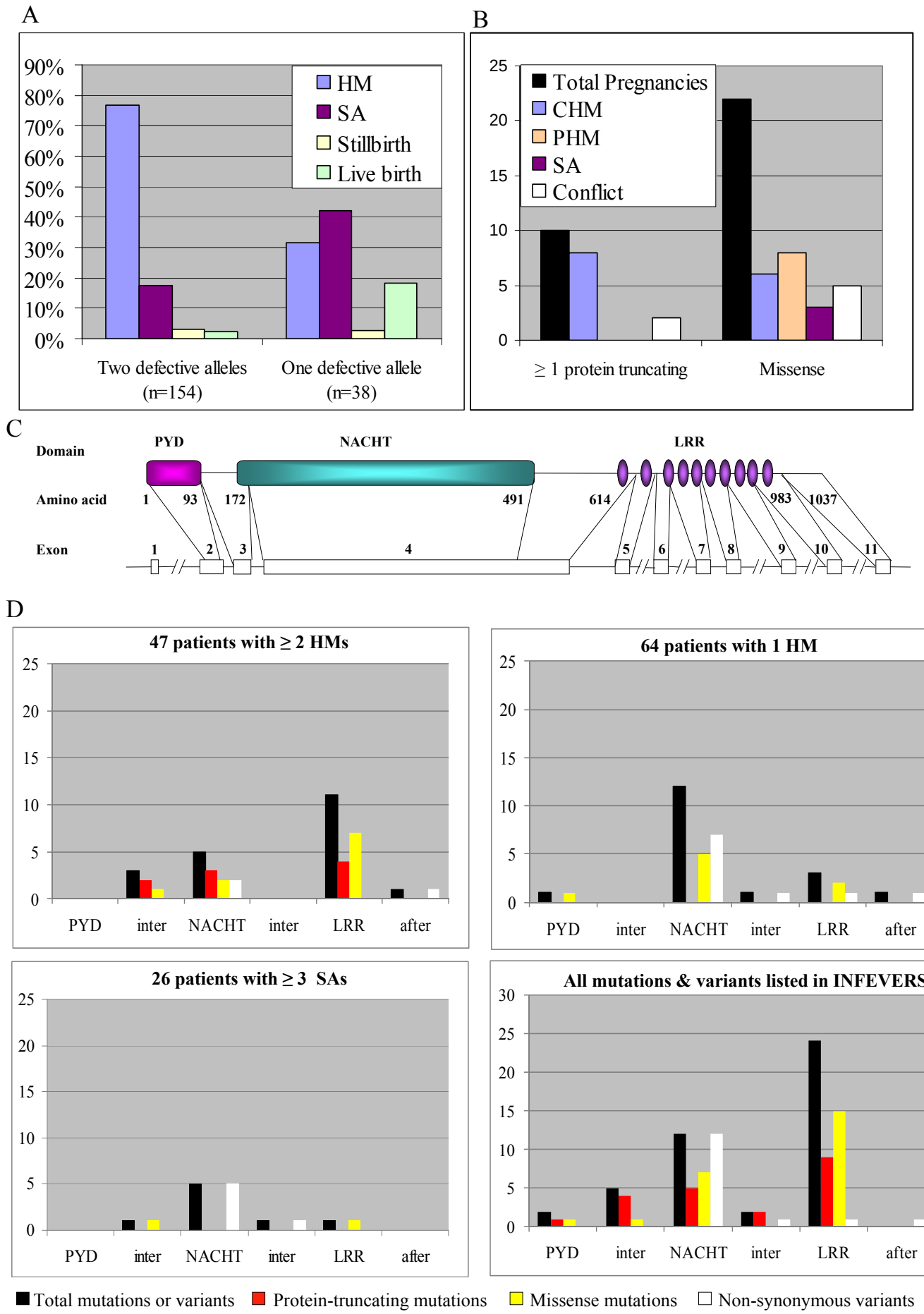
				eccentrically		
MoCa186	758	p.[V319I;G487E];[V319I;G487E];[F250L(;)A481T]	3	GA 40 5/7 w, birth weight 3670 g, female, spontaneous vaginal delivery, placenta 20x20, cord 50 cm with 3 vessels, central insertion	Very minimal chorangiosis and focal decidual necrosis.	Chorangiotic changes identified, subamniotic cyst
			2	GA 38 2/7 w, birth weight 4045 g, placenta weight 650 g, cord 53 cm and 1.2 cm, 3 vessels	Minimal chorangiosis and mild chorioamnionitis.	Oedematous and dysmature stem villi, chorangiosis

N, indicates the number of available histopathology blocks and slides; GA, stands for gestational age; w, indicates week. Abnormal features are underligned.

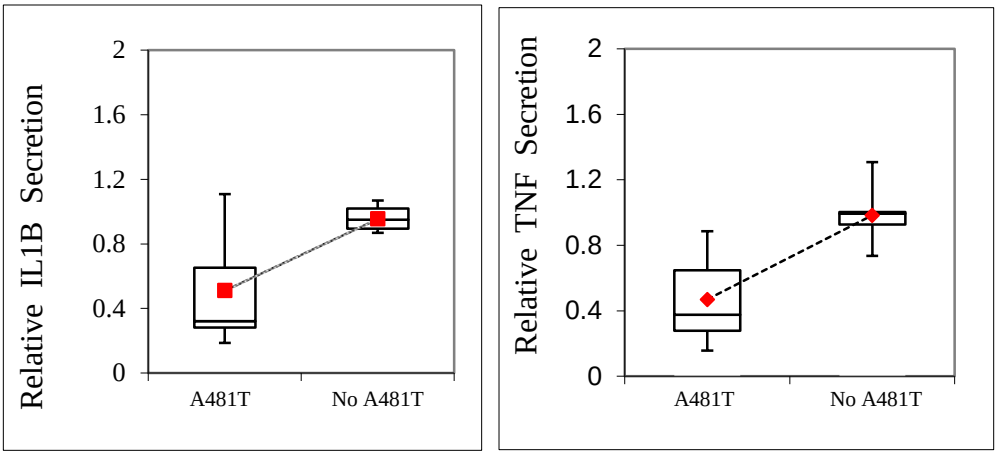
A



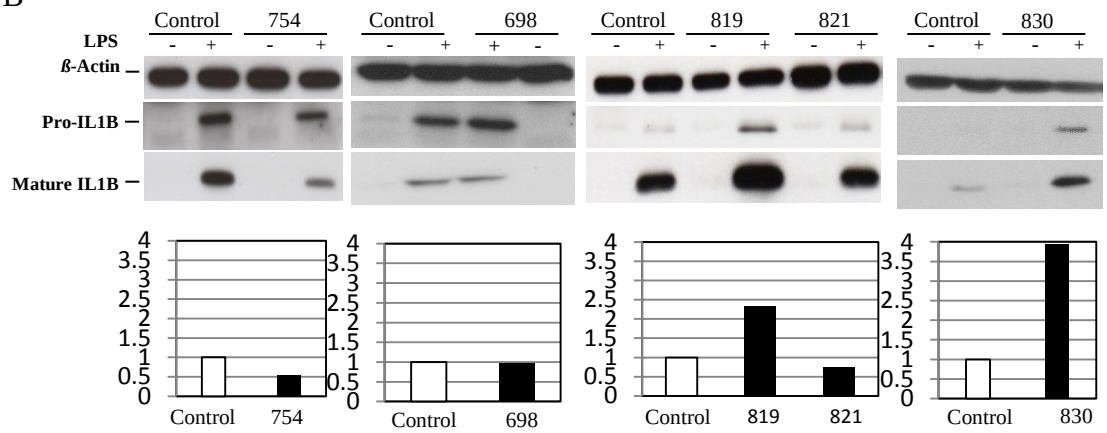
45 patients with ≥ 2 HMs **64 patients with 1 HM** **26 patients with ≥ 3 SAs**


A
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
HM
SA
Stillbirth
Live birth
Two defective alleles (n=154)
One defective allele (n=38)
B
25
20
15
10
5
0
Total Pregnancies
CHM
PHM
SA
Conflict
≥ 1 protein truncating
Missense
C
Domain
Amino acid
Exon
PYD
NACHT
LRR
1
93
172
491
614
983
1037
1
2
3
4
5
6
7
8
9
10
11
D
47 patients with ≥ 2 HMs
64 patients with 1 HM
26 patients with ≥ 3 SAs
All mutations & variants listed in INFEVERS
PYD
inter
NACHT
inter
LRR
after
Total mutations or variants
Protein-truncating mutations
Missense mutations
Non-synonymous variants

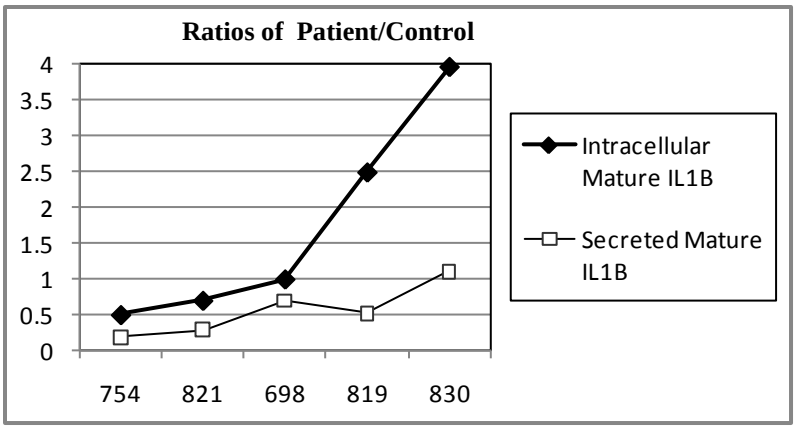
A


Relative IL1B Secretion
2
1.6
1.2
0.8
0.4
0
A481T
No A481T
Relative TNF Secretion
2
1.6
1.2
0.8
0.4
0
A481T
No A481T

B


Control
754
Control
698
Control
819
821
Control
830
LPS
ß-Actin
Pro-IL1B
Mature IL1B
Control
754
Control
698
Control
819
821
Control
830

C


Ratios of Patient/Control
4
3.5
3
2.5
2
1.5
1
0.5
0
754
821
698
819
830
Intracellular Mature IL1B
Secreted Mature IL1B

Supplementary table 1. Number of screened control chromosomes from different ethnic groups for the various mutations and variants

New mutation	Ethnic origin of the patient	Women of European descent with 5 to 16 children	Lebanese (general population)	Chinese (general population)	Pakistani (general population)	African American (general population)	Total number of controls
c.750C>A, p.Phe250Leu	African/Indian	208	42		76		326
c.930_931delGC, p.Q310HfsX38	Caucasian	208		98	-	-	306
c.1018_1020delGAGinsCAAAA, p.Glu340GlnfsX11	Caucasian	208		98	-	-	306
c.1018G>A, p.Glu340Lys	Pakistani	208		98	76	-	382
c.2444G>A, p.Arg815His	Algerian	220		-	-	-	220
c.2791_2792delTG, p.Cys931X	Caucasian	208		94	76	-	378
c.1169G>A, p.Arg390His	Guadeloupean	208		98	76		174
c.1237C>T, p.Arg413Trp	Haitian	208		98	76		174

Supplementary table 2. New mutations/variants and reproductive outcomes of the patients

Family ID	Patient ID	*NLRP7*	Reproductive Outcomes of the patients
MoUs167	712	p.[P716A];[Cys931X]	SA, PHM, PHM
MoNz170	725	p. [Q310HfsX38;A481T];[R693W]	CHM, SA, CHM, HM
MoCa179	744	p.[Glu340GlnfsX11];[R693W]	HM, BO, 3 SA, 2 HM
MoCa186	758	p.[V319I;G487E];[V319I;G487E];[F250L(;)A481T]	LB, CHM, LB
MoPa214	814	p.[V319I(;)E340K]	SA, SA, PHM (uterus retroverted, small leiomyoma)
MoGu248	897	p.[A481T];[A481T];[V319I(;)R390H(;)G487E]	ET, ET, SA, CHM, SA, SA, SA-CC, NP
MoHa259	919	p.[V319I(;)R413W(;)G487E]	NP, PHM-GTN (I-5)
MoAl17	M36	p.[G487E(;)R815H]	HM no other data

LB, stands for live birth; HM, for hydatidiform mole; PHM, partial HM; CHM, complete HM; SA, spontaneous abortion; BO, blighted ovum; ET, elective termination; CC, choriocarcinoma; GTN, gestational trophoblastic neoplasia grade I-5 according to HUPO nomenclature. New mutations are in red.

Supplementary table 3. Protein-truncating mutations in familial versus singleton cases

References	Familial cases		Singletons	
	N. of Families	≥1 Protein-truncating	N. of singleton	≥1 Protein-truncating
Murdoch et al. 2006	4	2	1	0
Qian et al., 2007	1	1		
Kou et al., 2008	3	2	5	3
Puechberty et al., 2008			1	0
Deveault et al., 2009	3	1	8	0
Hayward et al., 2009	5	3	8	2
Wang et al., 2009	7	3	13	5
Current study			5	3
Total	23	12	41	13
Protein-truncating	**52.17%**		**31.71%**	

N, stands for number.

Supplementary table 4. Non synonymous variants and reproductive hitories of patients of European descent

Family ID	Patient ID	Reproductive history by chronological order	Non synonymous changes
MoPa25	M4	HM no other data	p.[V319I]+[=]
MoPa26	M6	HM no other data	p.[=]+[=]
MoPa27	M20	HM no other data	p.[=]+[=]
MoCr51	399	2 HM-Lung adenocarcinome-died	p.[A481T]+[=]
MoCa58	433	HM, NP	p.[V319I]+[V319I]
MoCa63	700	NP, HM, NP	p.[=]+[=]
MoSw67	466	PHM, NP, 5 HM, HM	p.[=]+[=]
MoUs79	539	1 CHM, 4 IUI, 1 IVF, 1NP	p.[V319I]+[=]
MoCa80	540	1 CHM and multiple failed IVF	p.[V319I]+[=]
MoAu81	541	4 HM, 10 SA	p.[V319I]+[=]
MoCa115	701	PHM-GTN	p.[V319I]+[=]
MoFr119	M264	2 PHM/SA	p.[V319I(+)A481T]
MoFr121	M116	1 NP, 1 HM	p.[=]+[=]
MoCa168	747	infertility, clomiphene-HM-GTN (RP 2 D&C)	p.[G487E]+[=]
MoSw177	741	HM, EP, now pregnant	p.[Q310R;L311I;A481T]+[=]
MoCa178	742	HM	p.[=]+[=]
MoGe181	753	HM	p.[=]+[=]
MoCa182	754	NP, twin (HM+dead fetus), EA (RP 2 D&C)	p.[A481T]+[=]
MoCa189	764	SA, SA, SA-GTN	p.[A481T]+[=]
MoCa205	799	SA (RP requiring 3 D &C), BO	p.[A481T]+[=]
MoCa207	802	NP, SA, CHM-GTN(I-3) (2 D&C)	p.[G487E]+[=]
MoCa210	806	SA, SA, NP, NP, SA, NP, SA, SA	p.[V319I(+)A481T]
MoCa215	815	SA, SA, SA, SA, Pregnant	p.[=]+[=]
MoCa216	817	SA, SA, CHM (2 D&C), NP-CC	p.[=]+[=]
MoCa217	818	BO, NP[#], EP, BO, BO	p.[=]+[=]
MoIt218	819	BO, BO, NP, SA, SA	p.[A481T]+[=]
MoCa220	821	SA, SA (RP 2 D&C), SA	p.[Q310R(+)L311I(+)A481T]
MoCa223	830	SA, SA, SA, SA, EP, EP	p.[A481T]+[=]
MoCa224	831	NP, SA, SA, SA, SA, SA, SA, SA (RP 2 D&C)	p.[V319I]+[=]
MoCa228	840	CHM-GTN (I.4), pregnant	p.[G487E]+[=]
MoFr229	842	HM, HM	p.[=]+[=]
MoIt230	846	SA, CP, CP, CP, NP, SA , Pregnant	p.[V319I]+[=]
MoCa231	847	SA, SA, SA, Pregnant	p.[=]+[=]
MoCa232	856	TA, SA, SA, SA, Pregnant	p.[=]+[=]
2353	3	NP, SA, PHM, CHM	p.[K511R]+[=]
2364	3	2 NP, PHM, CHM	p.[V319I]+[=]
2546	3	PHM, NP, EA, PHM	p.[G487E]+[=]
2576	3	3 SA, 2 PHM (order unknown)	p.[G487E]+[=]
2805	3	3 SA, NP, 4 SA	p.[V319I(+)A481T]
2806	3	PHM, CHM	p.[=]+[=]
2926	3	4 SA, SB with pseudo molar placenta	p.[V319I]+[=]
2992	3	CHM, SB *(22w), IUFD (24 w, IUGR and malformations[#]), LB (33 w)	p.[A481T]+[=]
3914	3	HM, 3NP	p.[A481T]+[=]
3915	3	HM, 2NP	p.[=]+[=]
MoCa88	565	2 NP, 7 SA	p.[K511R]+[=]
MoCa234	864	1st pregnancy CHM	p.[=]+[=]
MoGr 235	871	SA, SA, SA, NP, SA, PR	p.[V319I(+)A481T]
MoNz238	875	PHM, SA, SA	V319I, M427T, F430L
MoCa239	880	SA, SA, SA, SA, HM-GTN	p.[=]+[=]
MoCa240	881	TA, SA, NP, PHM, SA, NP	p.[V319I]+[=]
MoIt241	882	NP, TA, NP, NP, HM-GTN	p.[K511R];[=]
MoIg243	885	SA, SA, SA, SA, SA	p.[G487E]+[=]
MoCa244	887	SA, SA, SA, SA, SA, SA, SA, Pregnant	p.[=];[=]

*The choronological order of the different pregnancies is unknown. CHM, indicates complete HM; PHM, partial HM; SA, spontaneous abortion; BO, blighted ovum; EP, ectopic pregnancy; EA, elective abortion; END, early neonatal death; CP, chemical pregnancy documented by a hormonal test; NP, normal pregnancy; IVF, in-vitro fertilization; IUI, intrauterine insemination; CC, choriocarcinoma; IUFD, intra uterine fetal demise; RP, retained placenta; D&C, dilatation and curettage * maternal hypertension; # the reported malformations are hypolastic left forearm, agenesis of the left hand, and micropenis.

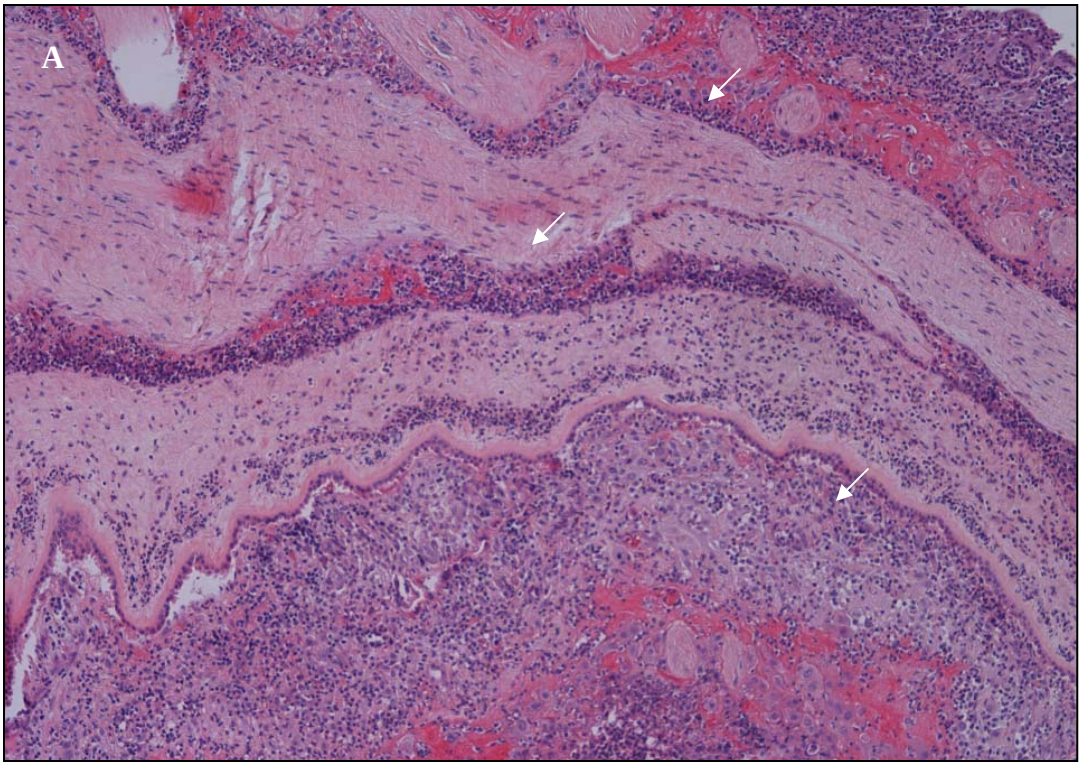
Suplementary table 5. Description provided by the gynecolgists about 4 stillbirths from patients with *NLRP7* mutations

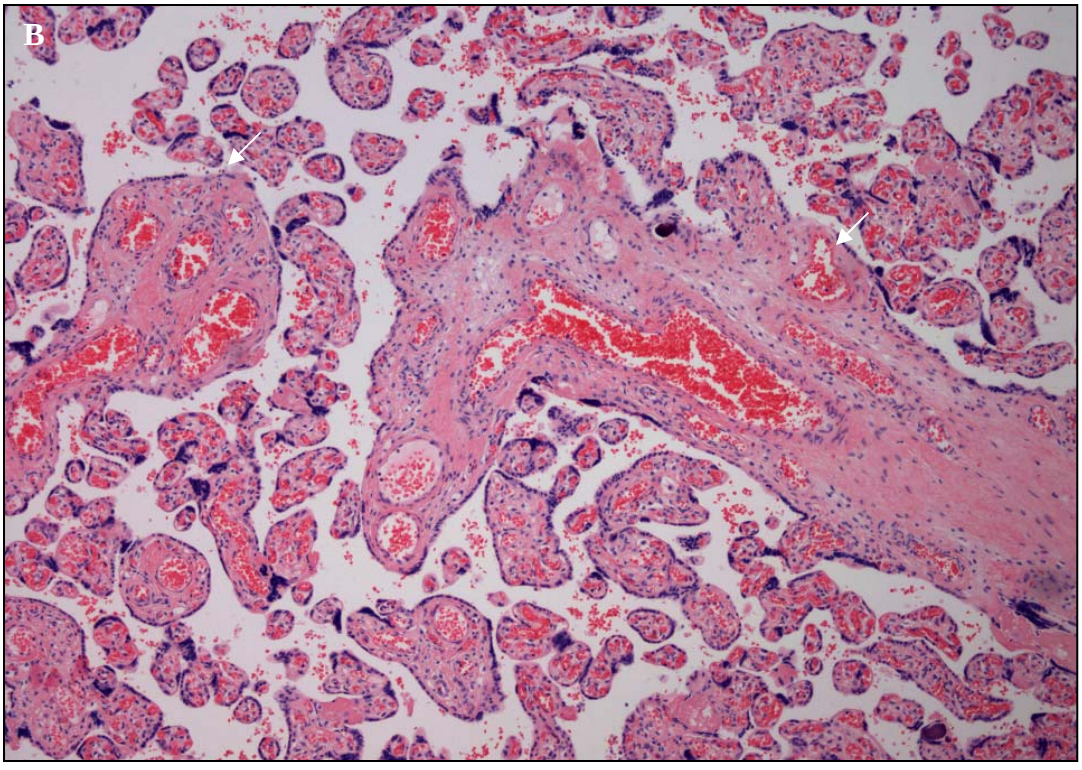
Family ID	Patient ID	Mutations	Description by the patient and available medical record
MoLb1	4	p.[G118X];[G118X]	According to the patient: GA 28 w, no preeclampsia, no bleeding.
		p.[G118X];[G118X]	According to the patient: GA full term pregnancy, home delivery of a baby 3.5 kb after uncomplicated pregnancy. The baby died one week later after suddening turning blue and being hypoxic for no apparent reason.
MoIn68	474	p.[R693P];[R693P]	According to the patient: GA full term pregnancy, vaginal delivery of a dead baby with no obvious mophological malformations. No preeclampsia during the pregnancy.
MoIn109	691	p.[N913S];[N913S]	According to the patient: GA 32 w.

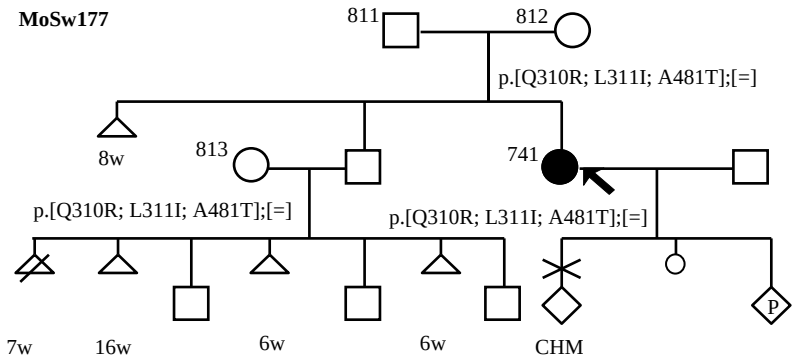
GA indicates gestational age; w, indicates weeks

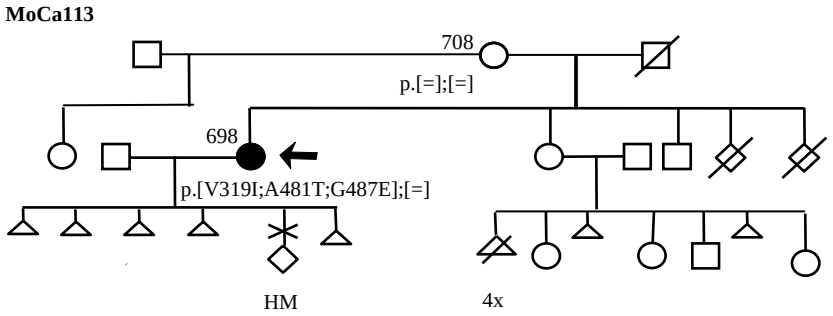
Supplementary table 6. Available medical information from 2 stillbirths from patients with *NLRP7* mutations

Family ID	Patient ID	Mutations	Description by the patient and available medical record
MoLb1	6	p.[G118X];[G118X]	Medical record: GA 26 w. Preterm labor and vaginal bleeding. The patient had preeclampsia with severe placental abruption. She then delivered a live male of 450 g (small for 26 w) who died later. Histopathology of the palcenta revealed normal decidua and villi with areas of infarction and calcification.
MoBa169	723	p.[G380R];[=]	Medical record: GA 29 w. Clinical manifestation: lower abdominal pain, reduced foetal movement for 7 days. Ultrasonography: Intra uterine fetal demise, no amniotic fluid was seen. There was a posterior haematoma in the placenta.Vaginal delivery of a morphologically normal dead fetus.


A


B





Ectopic pregnancy

Spontaneous abortion

Elective termination

Hydatidiform mole, complete or partial

Supplementary figure 2