Rare genetic variants suggest dysregulation of signaling pathways in low- and high-risk patients developing severe ovarian hyperstimulation syndrome

L. Borgwardt¹ · K. W. Olsen² · M. Rossing¹ · R. Borup Helweg-Larsen³ · M. Toftager⁴ · A. Pinborg⁵ · J. Bogstad⁴ · K. Løssl⁴ · A. Zedeler⁴ · M. L. Grøndahl²

Received: 11 June 2020 / Accepted: 7 September 2020 / Published online: 18 September 2020
© Springer Science+Business Media, LLC, part of Springer Nature 2020

Abstract

Purpose To investigate if rare gene variants in women with severe ovarian hyperstimulation syndrome (OHSS) provide clues to the mechanisms involved in the syndrome.

Methods Among participants in a prospective randomized study (Toftager et al. 2016), six women with predicted low and six women with predicted high risk of OHSS developing severe OHSS (grades 4 and 5, Golan classification) were selected. In the same cohort, six plus six matched controls developing no signs of OHSS (Golan grade 0) were selected. Whole-exome sequencing was performed. Analysis using a predefined in silico OHSS gene panel, variant filtering, and pathway analyses was done.

Results We found no convincing monogenetic association with the development of OHSS using the in silico gene panel. Pathway analysis of OHSS variant lists showed substantial overlap in highly enriched top pathways (p value range $p < 0.0001$ and $p > 9.8E-17$) between the low- and high-risk group developing severe OHSS, i.e., “the integrin-linked kinase (ILK) signaling pathway” and the “axonal guidance signaling pathway,” both being connected to vasoactive endothelial growth factor (VEGF) and endothelial function.

Conclusion Rare variants in OHSS cases with two distinct risk profiles enrich the same signaling pathways linked to VEGF and endothelial function. Clarification of the mechanism as well as potentially defining genetic predisposition of the high vascular permeability is important for future targeted treatment and prevention of OHSS; the potential roles of ILK signaling and the axonal guidance signaling need to be validated by functional studies.

Keywords Ovarian hyperstimulation syndrome · Pathway analyses · ILK signaling pathway · Axonal guidance signaling

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s10815-020-01941-0>) contains supplementary material, which is available to authorized users.

✉ L. Borgwardt
Line.gutte.borgwardt@regionh.dk

¹ Centre for Genomic Medicine, Copenhagen University Hospital Rigshospitalet, Copenhagen, Denmark

² Department of Obstetrics and Gynaecology, Fertility Clinic, Copenhagen University Hospital, Herlev, Denmark

³ Functional Genomics and Reproductive Health Group, Faculty of Health and Medical Sciences- Copenhagen University, Copenhagen, Denmark

⁴ Department of Obstetrics and Gynaecology, Fertility Clinic, Copenhagen University Hospital, Hvidovre, Denmark

⁵ Fertility Clinic, Copenhagen University Hospital Rigshospitalet, Copenhagen, Denmark

Introduction

Ovarian hyperstimulation syndrome (OHSS) is a complication to assisted reproductive technology (ART) treatment developing in the early luteal phase and/or early pregnancy. The clinical manifestations vary from abdominal distention and discomfort to potentially life-threatening, massive ovarian enlargement, and increased vascular permeability [1]. The mild forms of OHSS are common and occur in up to 30% of all IVF cycles [2–4], while more severe forms of OHSS occurs in 0.5–5.0% of IVF cycles [2, 5]. The severe OHSS is a systemic disorder including high vascular permeability correlated to an excessive ovarian response following ovarian stimulation (OS) and ovulation trigger with human chorionic gonadotrophin (hCG). Severe OHSS is rarely seen in women with a low ovarian response to OS and extremely rare in spontaneous cycles. The main theories explaining the mechanism of occurrence of

spontaneous OHSS, usually reported during pregnancy, are hypersecretion of thyroid-stimulating hormone and hCG or mutation of follicle-stimulating hormone (FSH) receptor [6, 7].

The etiology and pathophysiology of severe OHSS remain unclear. OHSS seems to involve an excessive secretion of vasoactive substances after the administration of hCG for triggering final oocyte maturation, involving vasoactive endothelial growth factor (VEGF) in the high vascular permeability with extravasation of fluid to the third space [8]. The longer half-life of hCG as compared with luteinizing hormone (LH) significantly contributes to the induction and development of OHSS and if pregnancy is established, the endogenous hCG from the syncytiotrophoblast potentiates the condition [9]. Therefore, primary preventive strategies aimed at avoiding the administration of hCG by the use of gonadotropin-releasing hormone agonist (GnRHa) to trigger ovulation in women with high ovarian response often combined with cryopreserving all embryos, significantly reducing the incidence of OHSS in ART [10]. VEGF has a central role in the syndrome. Blocking of the vasoactive endothelial growth factor receptor 2 (VEGFR2) by cabergoline has been suggested for prevention and treatment. However, a Cochrane analysis from 2017 concludes that there is low-quality evidence of cabergoline having a preventive effect on OHSS when administered around ovulation triggering or at oocyte pickup [11]. The role of VEGF in the syndrome has further been addressed by treatment with kisspeptin that inhibits the expression of VEGF with some effect on the symptoms [12].

Genetic variants in patients developing severe OHSS may provide further insight into the mechanisms involved in the syndrome. Known and frequent polymorphisms in genes predisposing to OHSS have been described (i.e., *FSHR*, *LHR*, *CYP11A*, *AMH*, *VEGFR2*) [13]. Recently, the VEGF receptor, *VEGFR3* (official name *FLT4*) has been suggested to have a role in OHSS. A case series performing whole-exome sequencing (WES) in four patients with severe OHSS after GnRHa trigger and freeze all, identified rare variants in this gene in two out of four patients [14]. Comparison of single-nucleotide polymorphisms (SNPs) in genes suggested to be involved in OHSS (*MTHFR*, *AMHR2*, *LHCGR*, *PGR*, *SERPINE1*) between 100 OHSS patients and 100 normal responders suggested that polymorphisms of *MTHFR*, *AMHR2*, and *LHCGR* genes could promote the risk of susceptibility to OHSS [13]. As overlap in variants in specific genes has been minor or absent in OHSS cases published, we here added a broader approach by also focusing on possible pathways influenced by the genetic variants.

We aimed to investigate if rare genetic variants in patients developing severe OHSS with predicted low and high risk of OHSS, respectively, provide clues to the mechanisms involved in the syndrome.

Material and methods

Patient population and selection

Among participants in a prospective randomized study (The Danish Ethics Committee, Capital Region of Denmark, Protocol #: H-B-2008-109) including 1050 women in first IVF cycle (2009–2013) [2], six women with predicted low risk of OHSS who developed severe OHSS (Golan classification grades 4–5) were selected [15]. In the same cohort, six matched controls (low risk, no OHSS, grade 0) were selected. Likewise, six women with predicted high risk of OHSS [15] who developed severe OHSS (grades 4 and 5, Golan classification) and six matched controls (no OHSS, grade 0) were selected. Criteria used to define low-risk and high-risk women were primarily the ovarian response, where low-risk and high-risk women were defined as below ten and above 15 oocytes retrieved, respectively. The controls were matched, in prioritized order, according to ovarian response (number of oocytes retrieved), age, diagnosis, estradiol level at the day of oocyte retrieval, and pregnancy (+/– positive hCG). See Table 1 and Online Resource 1. After oocyte retrieval, cumulus cells were isolated and frozen until analysis.

Ethical approval

Prior to the study, informed consent to use the biological material for further research was obtained from each of the patients (Biobank, Protocol #: H-B-2008-109). The collection of information and handling of patient data have been handled in accordance with the Danish Law on Processing of Personal Data (protocol #: H-B-2008-109) and approved by the Danish Health and Medicines Authority (EudraCT #: 2008-005452-24). Registration ID on [ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT00756028): NCT00756028).

Methods and DNA processing

The flow of the analyses performed in this study is summarized in Fig. 1. The analyses were divided into two parts;

- Analysis I: First, an analysis using a predefined in silico gene panel associated with OHSS was performed to investigate if rare variants involved in the development of OHSS were present among the cases developing OHSS.
- Analyses II and III: Second, pathway analyses including rare variants selectively present in the OHSS cases were performed to investigate potential pathways involved in the development of OHSS.

Table 1 Clinical and demographic characteristics in cases developing OHSS (+OHSS) and matched controls (−OHSS) having respectively low-risk and high-risk profiles for developing the syndrome

	Low-risk +OHSS	Low-risk −OHSS	High-risk +OHSS	High-risk −OHSS	Low-risk one- way ANOVA <i>p</i> value (+OHSS vs. −OHSS)	High-risk one- way ANOVA <i>p</i> value (+OHSS vs. −OHSS)
Number	6	6	6	6	-	-
Age (years)	31.7 ± 4.7	31.1 ± 4.7	31.3 ± 4.9	28.8 ± 2.7	0.8579	0.3018
BMI (kg/m ²)	23.7 ± 5.3	23.5 ± 3.6	23.3 ± 2.4	23.3 ± 2.8	0.9405	0.9828
Primary cause of infertility						
- Female factor	2	3	1	4	-	-
- Male factor	2	2	4	2	-	-
- Unexplained	1	1	1	-	-	-
- Other causes*	1	-	-	-	-	-
OHSS score						
- Grade 4	3	-	-	-	-	-
- Grade 5	3	-	6	-	-	-
- No OHSS	-	6	-	6	-	-
Treatment characteristics						
Protocol						
- Short	3	4	1	3	-	-
- Long	3	2	5	3	-	-
Total FSH dose (IU)	2000 ± 1069	1908 ± 828	1963 ± 521	1433 ± 211	0.8714	0.0437
Stimulation duration (days)	10.5 ± 3.9	10.3 ± 2.7	11.8 ± 1.5	9.7 ± 1.5	0.9332	0.0304
Estradiol at OPU (nmol/l)	3.4 ± 1.8	2.9 ± 1.1	12.7 ± 5.1	5.3 ± 1.6	0.5882	0.00684
Cycle characteristics						
No. of oocytes	7 ± 1.3	6.6 ± 1.1	22.7 ± 5.6	18.3 ± 3.8	0.5987	0.1479
No. of 2PN zygotes	3.7 ± 1.2	3.2 ± 1.5	11.5 ± 4.0	6.7 ± 4.0	0.579	0.0649
No. of high-quality embryos	1 ± 0.5	1 ± 0.6	1 ± 0	1 ± 0	1	0.3409
No. of transferred embryos	1.3 ± 0.5	1.2 ± 0.4	1 ± 0	1 ± 0	0.549	0.3409
Outcome						
Positive hCG (n/n) (%)	4/6 (66.7)	3/6 (50)	4/6 (66.7)	4/6 (66.7)	-	-
No. of clinical pregnancies**	2	2	4	2	-	-

Mean ± standard deviation

*Single women and women with a female partner

**Fetal heartbeat in gestational weeks 6–7. OHSS, ovarian hyperstimulation syndrome; FSH, follicle-stimulating hormone; BMI, body mass index; OPU, oocyte pickup; 2PN, two-pronuclear; hCG, human chorionic gonadotropin

Oocyte pickup

During the oocyte pickup procedure for each woman, cumulus cells were collected from one cumulus-oocyte

complex originating from a ≥ 17 mm follicle. Cumulus cells were transferred to 500 μ l RNase inhibitor PBS and frozen at -20 °C and transferred to -80 °C for long-term storage.

Fig. 1 The flow and the results of the analyses performed in this study are summarized in the figure. Analysis I: The predefined in silico gene panel including 76 genes predisposing to OHSS was analyzed for the 12 cases developing OHSS. Analysis II: Six patients from the low-risk +OHSS group were destined to be cases and the six patients from the low-risk -OHSS group were destined to be controls. Analysis III: Six patients from the high-risk +OHSS group were destined to be cases and the six patients from the high-risk -OHSS group were destined to be controls. OHSS: Ovarian hyperstimulation syndrome; +OHSS: women who developed OHSS; -OHSS: women who did not develop OHSS

Whole-exome sequencing

Whole-exome sequencing was performed on DNA extracted from cumulus cells from each of the 24 women selected for the study. In brief, 500 ng of DNA was fragmented to an average size of 300 base pairs using Covaris S2 (Woburn, MA, USA). Adaptor ligation was performed using the KAPA HTP Library Preparation Kit (Roche Diagnostics, Basel, Switzerland) on a Sciclone G3 robot (Perkin Elmer, Waltham, MA, USA). Sequence capture targeting the exome was done with the SureSelectXT Clinical Research Exome kit (Agilent) and followed sequencing on HiSeq2500 or NextSeq500 (Illumina, San Diego, CA, USA).

Data processing

Genetic variants were called using the GATK 4.1 pipeline with a minimum of 10-fold read coverage. Variant filtering and pathway analyses were done using the Ingenuity Variant Analysis tool (Qiagen Bioinformatics, Redwood City, CA), excluding the low-quality scored variants. Only variants with a minor allele frequency of less than 0.5% in 1000 genome and ExAC data and variants in coding regions (+/- 6 bp) were selected for further analysis. Synonymy variants were excluded unless reported in the Human Gene Mutation Database (HGMD) or ClinVar database.

Analysis I: In silico gene panel

To investigate if rare variants potentially involved in the development of OHSS were present, variants called in the 12 cases with OHSS were filtered using a predefined in silico gene panel including 76 genes predisposing to OHSS (Online Resource 2). The genes included in the gene panel were selected based on an extensive literature search in PubMed and Online Mendelian Inheritance in Man (OMIM) and were implicated in controlled ovarian stimulation response, associated with OHSS or regulation of gonadotropin action and/or ovarian angiogenesis [3, 14, 16–24]. The gene panel included both known disease-associated genes related to OHSS and candidate genes which association with OHSS needs further study. The gene panel was created during June 2018.

All variants called by the in silico gene panel were visually validated in Integrative Genomics Viewer (IGV 2.4.9); only classes 3, 4, and 5 variants were included [25].

Analyses II and III: Pathway analyses

The case/control setup for the WES was as followed:

Six patients from the low-risk +OHSS group were destined to be cases and the six patients from the low-risk -OHSS group were destined to be controls (analysis II).

Six patients from the high-risk +OHSS group were destined to be cases and the six patients from the high-risk –OHSS group were destined to be controls (analysis III).

Thus, the setup included variants which were identified in cases but not identified in controls in the low-risk group and high-risk group, respectively. The called variants were submitted to pathway analysis. Top enriched pathways were identified and ranked according to their significance to our input data. Furthermore, overlapping genes between the two risk groups were submitted to overrepresentation analysis in the statistical software R [26].

Statistical methods

For the pathway analyses, Fisher's exact test was performed using the Ingenuity Variant Analysis tool, $p < 0.05$. Furthermore, gene ontology gene sets of biological function from the Molecular Signatures Database (<https://www.gsea-msigdb.org/gsea>, Broad Institute, San Diego, CA, USA) were used to test for significantly enriched gene ontology terms in the 466 rare variants found in genes common for +OHSS from the two risk groups. Enrichment was determined by the one-sided version of Fisher's exact test, $p < 0.001$ (q value < 0.05) [27], as implemented in the ClusterProfiler R package [28].

Results

Whole-exome sequencing (WES) was performed on all 24 women included in the study. Table 1 shows bibliographic and treatment data of the women included in the study, grouped in low and high risk; Online Resource 1 shows the data merged.

In silico gene panel

Screening for rare variants using the in silico OHSS gene panel resulted in seven variants identified in four cases from the low-risk OHSS group and three cases from the high-risk group. The variants are listed in Table 2. The variants comprise five missense variants, one duplication, and one start loss variant. According to the American College of Medical Genetics (ACMG) guidelines, all seven variants were classified as variants of unknown significance (class 3) [25].

Pathway analyses

In the low-risk group, 2824 rare variants called in 2672 genes were present in the six cases developing OHSS (low-risk +OHSS) and absent in the six controls who did not develop OHSS (low-risk –OHSS). In the high-risk group, 2242 rare variants called in 2215 genes were present in the six cases

developing OHSS (high-risk +OHSS) and absent in the six controls who did not develop OHSS (high-risk –OHSS) (Fig. 1). Venn analysis showed an overlap in 466 (10%) genes between the two lists (Online Resource 3). The number of cases representing rare variants in the overlapping genes were low, primarily two to three cases per gene or one to two per group.

The potential pathways involved in the development of OHSS were investigated in analyses II and III. The variants enriched in the low-risk group and in the high-risk group, respectively, were submitted to pathway analysis.

The pathways for the low-risk group and the high-risk group are listed in Online Resource 4 and 5 respectively. In the low-risk group, 99 pathways were enriched ($p < 0.01$); in the high-risk group, 59 pathways were enriched ($p < 0.01$). The Venn analysis shows the overlap among the 39 pathways (Online Resource 6). The overlapping pathways most significantly enriched (p value between 0.0001 and $9.8E-17$) and, by the authors' selected pathways, are presented in Table 3. Genes included in the pathways presented in Table 3 are listed in Online Resource 7.

When submitting the 466 genes overlapping between cases in the two risk groups to enrichment analysis, the top hallmark gene set enriched were “complement” (genes encoding components of the complement system, a part of the innate immune system) and “interferon-gamma response” ($p = 0.001$) (Online Resource 8). Among the top biological process was “cell-cell adhesion” (genes encoding products involved in the attachment of a cell to another cell) ($p = 4.1E-18$) and “activation of the innate immune response” ($p = 8.2E-06$) (Online Resource 9). The summary of these results is shown in Fig. 2.

Discussion

In the first part of this study, we performed a screening of the in silico OHSS gene panel in the 12 cases developing OHSS to explore if rare genetic variants predispose to OHSS development. As previous studies aiming to identify monogenetic explanations for developing OHSS, we found limited overlap with previously published cases. This indicates and supports that monogenetic predisposition to the development of OHSS is limited, making the pathway analyses performed intriguing. Our results of the pathway analyses showed that compared with controls, the WES of cases developing severe OHSS with a predisposed low- or high-risk profile, respectively, suggests dysregulation of signaling pathways that may reflect the etiology and perhaps even pathophysiology of the syndrome. Details about the dysregulated pathways in OHSS could provide more targeted tools to prevent and treat OHSS.

Interestingly, we identified a substantial overlap between highly significantly enriched pathways of the rare variants found in the low and the high risk among women who

Table 2 Rare variants identified in cases developing severe OHSS using the OHSS in silico gene panel

Gene	NM_number	dbSNP	Nucleotide	Amino acid	Previously reported in the literature	Translation impact	GnomAD*	Risk group**	Classification***
<i>CASP9</i>	NM_001229	rs146054764	c.710A>C	p.(His237Pro)	Yes	Missense	0.14%	High	Class 3
<i>CASP9</i>	NM_001229	NA	c.52G>A	p.(Glu18Lys)	No	Missense	****	Low	Class 3
<i>OCN</i>	NM_002538	rs761617848	c.363_368dup	p.(Tyr128_Gly129dup)	No	In frame	0.00%	High	Class 3
<i>ESR1</i>	NM_0011227-42	rs77797873	c.896A>G	p.(Lys299Arg)	No	Missense	0.09%	High	Class 3
<i>AMHR2</i>	NM_020547	rs375138212	c.1120G>A	p.(Gly374Ser)	No	Missense	0.01%	Low	Class 3
<i>CYP11A1</i>	NM_000781	rs139449608	c.992C>T	p.(Thr331Met)	No	Missense	0.00%	Low	Class 3
<i>CGB1</i>	NM_033377	rs1201548329	c.1A>T	p.?	No	Start loss	0.00%	Low	Class 3

*GnomAD: genome aggregation database (<https://gnomad.broadinstitute.org/>)

**Risk group: the OHSS risk group in which the variant was observed

***Classified according to the American College of Medical Genetics (ACMG) guidelines (Richards, et al. 2015)

****Not reported in gnomAD. NA, not applicable. All variants are variants of uncertain significance (class 3)

developed severe OHSS (Table 3a), of which several are reported to influence vessel function. The axonal guidance signaling known to be involved in angiogenesis and endothelial function [36] was among the overlapping pathways most significantly enriched. Genes with variants in axonal guidance

signaling were genes coding for disintegrins and metalloproteinases (*ADAM8*, *11*, and *33*) and extracellular matrix (ECM)-modifying proteins (*ADAMTS3*, *4*, *9*, *13*, and *20*), Semaphorin (*SEMA6A*), and Plexins (*PLXNA3*, *PLXNB2*, *3*, and *PLXND1*). Factors connected to axonal guidance

Table 3 Overlapping pathways (A) highly enriched and selected (B) by rare gene variants in cases developing OHSS having respectively low-risk and high-risk profiles for developing the syndrome

Pathway name	Low-risk OHSS			High-risk OHSS		
	p value	#Genes	#Cases	p value	#Genes	#Cases
A: Highly enriched pathways						
Actin cytoskeleton signaling*	9.79E-17	47	6	3.58E-07	30	6
Axonal guidance signaling*	3.68E-16	67	6	6.35E-14	57	6
Protein kinase A signaling	2.13E-15	57	6	7.61E-06	36	6
Cardiac hypertrophy signaling (enhanced)	2.40E-15	66	6	1.32E-06	44	6
Synaptogenesis signaling pathway	3.62E-10	43	6	1.50E-11	41	6
GP6 signaling pathway*	2.01E-09	25	6	1.57E-08	22	5
Osteoarthritis pathway*	1.41E-08	34	6	2.06E-05	26	6
Molecular mechanisms of cancer	4.96E-08	49	6	1.20E-08	45	6
ILK signaling*	5.49E-06	27	6	4.98E-07	26	6
Corticotropin-releasing hormone signaling	1.37E-05	24	6	3.64E-07	24	6
Hepatic fibrosis/hepatic stellate cell activation	1.62E-05	26	6	1.27E-08	28	6
Cellular effects of sildenafil (Viagra)	2.36E-05	23	6	3.54E-09	26	6
Epithelial adherens junction signaling*	2.47E-05	22	6	4.77E-06	21	6
Gap junction signaling	2.93E-05	26	6	6.13E-05	23	6
B: Selected pathways						
Estrogen receptor signaling*	1.44E-05	21	6	1.12E-02	15	5
eNOS signaling*	2.97E-06	25	6	1.53E-02	17	6
Adrenomedullin signaling pathway*	1.51E-04	26	6	1.29E-02	20	6

*The variant genes enriching these pathways are shown in Online resource 7

Fig. 2 Signaling pathways and OHSS. Whole-exome analysis shows rare variants selectively present in women developing OHSS. Signaling pathways enriched by these rare variants in women having low-risk and high-risk profiles for developing the syndrome, respectively, show substantial overlap. This figure highlights some of the pathways and refers to potential involvement in the central pathophysiology of OHSS, the leaky endothelium. Superscript 1: Drey Mueller et al. [29]; superscript

2: Villasante et al. [30]; superscript 3: Jauhainen et al. [21] and Adams and Eichmann [31]; Superscript 4: Li and Zhan [32]; Superscript 5: Garcia-Jerez et al. [33]; Superscript 6: Umer et al. [18]; Superscript 7: Feletou et al. [34]; Superscript 8: Kosaka et al. [35]. *Biological function enriched by rare genetic variants overlapping between low-risk and high-risk cases with severe OHSS

signaling are influenced by the binding of VEGF to its receptors [21] supporting that this pathway could be involved in the pathophysiology of OHSS. During follicle growth, an increase in expression of genes included in the axonal guidance signaling pathway is seen [37]. Interestingly, a recent transcriptome study on cultured bovine granulosa cells stimulated with L-lactate reported that axonal guidance signaling was among the highest affected pathways in differentially expressed genes and estrogen as well as VEGF were suggested as potential upstream regulators [38]. L-Lactate in the follicular fluid increases at the time of LH surge and it is suggested as having a regulatory role in granulosa cell differentiation [39, 40]; however, if OHSS patients have altered intrafollicular, metabolism including L-lactate production is speculative.

Another highly enriched pathway overlapping between the two groups developing severe OHSS was the integrin-linked kinase (ILK) signaling pathway. As for axonal guidance, ILK signaling is central in the cell to ECM adhesion and signaling. ILK activation has been shown to promote VEGF expression in tumor cells and has an essential role in VEGF-mediated endothelial activation (Hannigan et al. 2005).

Interestingly, in 2019, Umer et al. identified ILK as a critical regulator of VEGFR3 signaling and lymphatic vascular

growth [18] and Stouffs et al. published a case report on WES, in four patients with severe OHSS after GnRHa trigger and freeze all, focusing on rare genetic variants, suggesting *VEGFR3* (official name *FLT4*), to play a role in OHSS [14] as two of four patients had a rare variant in this gene.

The GP6 (glycoprotein VI platelet) signaling pathway was additionally among the highly significantly enriched overlapping pathways. GP6, which is a member of the immunoglobulin superfamily, is expressed in platelets and their precursors (megakaryocytes). GP6 serves as the major signaling receptor for collagen, which leads to platelet activation and thrombus formation [32]. Whether this pathway is involved in the pathophysiology of OHSS and the following higher risk of thrombosis observed in OHSS patients need to be further elucidated.

In both OHSS groups, variant genes enriched the eNOS (endothelial nitric oxide synthase) signaling, a pathway essential for healthy vascular function [34]. High estradiol level has been shown to increase NO and VEGF levels in endothelial tissue in an OHSS model [12]. The present finding further supports a possible involvement of eNOS in the development of OHSS. eNOS is also central in the adrenomedullin signaling pathway which is also found in the overlapping list. Adrenomedullin is a vasoactive peptide, which is suggested to be involved in the rare and potentially life-threatening

vascular disorder, systemic capillary leak syndrome (SCLS) [41] resembling the vascular leakiness observed in severe OHSS. Adrenomedullin is produced in many tissues and has been found in the human pre-ovulatory follicle in the follicular fluid [20]. It is expressed in mural granulosa cells [42]; however, the local production seems without direct relevance for the development of OHSS as a recent study showed higher adrenomedullin expression in mural granulosa cells after GnRH α triggering as compared with hCG triggering [43].

In addition to the chosen broad approach focusing on possible pathways influenced by the genetic variants in each risk group, we submitted the variants in genes overlapping between the OHSS cases in the two groups. These genes did primarily enrich biological functions related to cell-cell adhesion in line with the highly significant pathways enriched in the +OHSS in both risk groups. Interestingly, the overlapping genes additionally enriched functions related to the activity of the innate immune response. The immune system has been suggested to be involved in the pathophysiology of OHSS [35, 44].

Our findings are based upon a small case-control study, which leads to a high risk of selection bias and confounding. Among the low-risk women, bibliographic, treatment, cycle, and outcome characteristics were all comparable between cases and controls. Among the high-risk women, there were some, relatively small treatment differences as the controls had a shorter duration of stimulation, lower total dose of FSH, and lower estradiol level at oocyte pickup. The higher estradiol level in the +OHSS high-risk women as compared with control despite the same number of oocytes retrieved is in accordance with known factors involved in developing OHSS (3). In contrast, estradiol level in the +OHSS in the low-risk group was not elevated as compared with control indicating a difference in the importance of estradiol in developing OHSS between the two risk groups, which needs to be elucidated further. As we primarily focus on the substantial overlap in enriched pathways in +OHSS between the low- and high-risk groups, these differences are considered to have a relative low impact on the conclusion. The filtering of variants used for pathway analyses is another challenging step at risk of including benign variants. But, based on the strict criteria for the variant filtering listed in the “Material and methods” section, we found the risk of including a benign variant in our pathway analyses low.

In summary, we found no convincing monogenetic association with the development of OHSS using the *in silico* gene panel; in the pathway analyses, we found substantial overlap in pathways highly enriched in OHSS women in both the low-risk and high-risk groups. Clarification of the mechanism as well as the potentially defining genetic predisposition of the high vascular permeability is important for future targeted treatment and prevention of OHSS; the potential role of ILK signaling, the axonal guidance signaling, and other overlapping

pathways in the development of OHSS need to be validated by functional studies.

Acknowledgments The authors would like to thank the staff at the Fertility Clinic, Copenhagen University Hospital, Rigshospitalet, for collecting and handling the samples and the staff at Centre for Genomic Medicine, Copenhagen University Hospital, Rigshospitalet, for performing the whole-exome sequencing analysis and the data processing.

Authors' contributions M.L.G. and R.B.H.L. initiated the study. L.B., M.L.G., M.R., and K.W.O. were involved in the design, in the analysis and interpretation of data, in the preparation of the figures and tables, and in writing and finalizing the manuscript. M.T., A.P., J.B., K.L., and A.Z. were involved in patient recruitment and sample collection and in finalizing the manuscript. R.B.H.L. was involved in finalizing the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethics approval The collection of information and handling of patient data have been handled in accordance with the Danish Law on Processing of Personal Data (protocol #: H-B-2008-109) and approved by Danish Health and Medicines Authority (EudraCT #: 2008-005452-24). Registration ID on [ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT00756028): NCT00756028.

Consent to participate and for publication Prior to the study, informed consent to use the biological material for further research was obtained from each of the patients (Biobank, Protocol #: H-B-2008-109).

References

1. Wu L, Sun Y, Wan J, Luan T, Cheng Q, Tan Y. A proteomic analysis identifies candidate early biomarkers to predict ovarian hyperstimulation syndrome in polycystic ovarian syndrome patients. *Mol Med Rep*. 2017;16(1):272–80. <https://doi.org/10.3892/mmr.2017.6604>.
2. Toftager M, Bogstad J, Bryndorf T, Lossel K, Roskaer J, Holland T, et al. Risk of severe ovarian hyperstimulation syndrome in GnRH antagonist versus GnRH agonist protocol: RCT including 1050 first IVF/ICSI cycles. *Hum Reprod*. 2016;31(6):1253–64. <https://doi.org/10.1093/humrep/dew051>.
3. Nasti CO, Teixeira DM, Moroni RM, Leitao VM, Martins WP. Ovarian hyperstimulation syndrome: pathophysiology, staging, prediction and prevention. *Ultrasound Obstet Gynecol*. 2015;45(4):377–93. <https://doi.org/10.1002/uog.14684>.
4. Smith V, Osianlis T, Vollenhoven B. Prevention of ovarian hyperstimulation syndrome: a review. *Obstet Gynecol Int*. 2015;2015: 514159. <https://doi.org/10.1155/2015/514159>.
5. Alptekin H, Gezgin K, Yilmaz FY. Bilateral megalocystic ovaries following in vitro fertilization detected during cesarean section: a case presentation. *J Turk Ger Gynecol Assoc*. 2012;13(2):142–4. <https://doi.org/10.5152/jtgga.2011.76>.
6. Casarini L, Crepieux P. Molecular mechanisms of action of FSH. *Front Endocrinol (Lausanne)*. 2019;10:305. <https://doi.org/10.3389/fendo.2019.00305>.
7. Dey AK, Dubey A, Mittal K, Kale S. Spontaneous ovarian hyperstimulation syndrome - understanding the dilemma. *Gynecol*

- Endocrinol. 2015;31(8):587–9. <https://doi.org/10.3109/09513590.2015.1037268>.
8. Gomez R, Soares SR, Busso C, Garcia-Velasco JA, Simon C, Pellicer A. Physiology and pathology of ovarian hyperstimulation syndrome. *Semin Reprod Med*. 2010;28(6):448–57. <https://doi.org/10.1055/s-0030-1265670>.
 9. Humaidan P, Nelson SM, Devroey P, Coddington CC, Schwartz LB, Gordon K, et al. Ovarian hyperstimulation syndrome: review and new classification criteria for reporting in clinical trials. *Hum Reprod*. 2016;31(9):1997–2004. <https://doi.org/10.1093/humrep/dew149>.
 10. Engmann L, Benadiva C, Humaidan P. GnRH agonist trigger for the induction of oocyte maturation in GnRH antagonist IVF cycles: a SWOT analysis. *Reprod BioMed Online*. 2016;32(3):274–85. <https://doi.org/10.1016/j.rbmo.2015.12.007>.
 11. Mourad S, Brown J, Farquhar C. Interventions for the prevention of OHSS in ART cycles: an overview of Cochrane reviews. *Cochrane Database Syst Rev*. 2017;1:CD012103. <https://doi.org/10.1002/14651858.CD012103.pub2>.
 12. Zhai J, Liu J, Zhao S, Zhao H, Chen ZJ, Du Y, et al. Kisspeptin-10 inhibits OHSS by suppressing VEGF secretion. *Reproduction*. 2017;154(4):355–62. <https://doi.org/10.1530/rep-17-0268>.
 13. Ghaderian SMH, Akbarzadeh R, Mohajerani F, Khodaii Z, Salehpour S. The implication of single-nucleotide polymorphisms in ovarian hyperstimulation syndrome. *Mol Reprod Dev*. 2019;86(8):964–71. <https://doi.org/10.1002/mrd.23171>.
 14. Stouffs K, Daelemans S, Santos-Ribeiro S, Seneca S, Gheldof A, Gurbuz AS, et al. Rare genetic variants potentially involved in ovarian hyperstimulation syndrome. *J Assist Reprod Genet*. 2019;36(3):491–7. <https://doi.org/10.1007/s10815-018-1372-5>.
 15. Shields R, Vollenhoven B, Ahuja K, Talmor A. Ovarian hyperstimulation syndrome: a case control study investigating risk factors. *Aust N Z J Obstet Gynaecol*. 2016;56(6):624–7. <https://doi.org/10.1111/ajo.12515>.
 16. Orkunoglu-Suer F, Harralson AF, Frankfurter D, Gindoff P, O'Brien TJ. Targeted single molecule sequencing methodology for ovarian hyperstimulation syndrome. *BMC Genomics*. 2015;16:264. <https://doi.org/10.1186/s12864-015-1451-2>.
 17. Ghasemi N, Dehghani Firouzabadi R, Ahmadi S. Association of -460C/T and +405 G/C polymorphisms of vascular endothelial growth factor gene and susceptibility to ovarian hyperstimulation syndrome. *Int J Reprod Biomed (Yazd)*. 2017;15(2):87–92.
 18. Urner S, Planas-Paz L, Hilger LS, Henning C, Branopolski A, Kelly-Goss M, et al. Identification of ILK as a critical regulator of VEGFR3 signalling and lymphatic vascular growth. *EMBO J*. 2019;38(2). <https://doi.org/10.15252/embj.201899322>.
 19. Delbaere A, Smits G, De Leener A, Costagliola S, Vassart G. Understanding ovarian hyperstimulation syndrome. *Endocrine*. 2005;26(3):285–90. <https://doi.org/10.1385/endo.26:3:285>.
 20. Guimera M, Morales-Ruiz M, Jimenez W, Balasch J. LH/HCG stimulation of VEGF and adrenomedullin production by follicular fluid macrophages and luteinized granulosa cells. *Reprod BioMed Online*. 2009;18(6):743–9. [https://doi.org/10.1016/s1472-6483\(10\)60021-1](https://doi.org/10.1016/s1472-6483(10)60021-1).
 21. Jauhainen S, Laakkonen JP, Ketola K, Toivanen PI, Nieminen T, Ninchoji T, et al. Axon guidance-related factor FLRT3 regulates VEGF-signaling and endothelial cell function. *Front Physiol*. 2019;10:224. <https://doi.org/10.3389/fphys.2019.00224>.
 22. Zhang L, Feng T, Spicer LJ. The role of tight junction proteins in ovarian follicular development and ovarian cancer. *Reproduction*. 2018;155(4):R183–98. <https://doi.org/10.1530/rep-17-0503>.
 23. Binder H, Dittrich R, Einhaus F, Krieg J, Müller A, Strauss R, et al. Update on ovarian hyperstimulation syndrome: part 1—incidence and pathogenesis. *Int J Fertil Womens Med*. 2007;52(1):11–26.
 24. Lorenzi D, Fernández C, Bilinski M, Fabbro M, Galain M, Menazzi S, et al. First custom next-generation sequencing infertility panel in Latin America: design and first results. *JBRA Assist Reprod*. 2020;24(2):104–14. <https://doi.org/10.5935/1518-0557.20190065>.
 25. Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, et al. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genet Med*. 2015;17(5):405–24. <https://doi.org/10.1038/gim.2015.30>.
 26. Core RCT. R: a language and environment for statistical computing. Vienna: R Foundation for Statistical Computing; 2019. <http://www.r-project.org/index.html>
 27. Boyle EI, Weng S, Gollub J, Jin H, Botstein D, Cherry JM, et al. GO::TermFinder—open source software for accessing Gene Ontology information and finding significantly enriched Gene Ontology terms associated with a list of genes. *Bioinformatics*. 2004;20(18):3710–5. <https://doi.org/10.1093/bioinformatics/bth456>.
 28. Yu G, Wang LG, Han Y, He QY. clusterProfiler: an R package for comparing biological themes among gene clusters. *Omics*. 2012;16(5):284–7. <https://doi.org/10.1089/omi.2011.0118>.
 29. Dreytmueller D, Pruessmeyer J, Groth E, Ludwig A. The role of ADAM-mediated shedding in vascular biology. *Eur J Cell Biol*. 2012;91(6–7):472–85. <https://doi.org/10.1016/j.ejcb.2011.09.003>.
 30. Villasante A, Pacheco A, Pau E, Ruiz A, Pellicer A, Garcia-Velasco JA. Soluble vascular endothelial-cadherin levels correlate with clinical and biological aspects of severe ovarian hyperstimulation syndrome. *Hum Reprod*. 2008;23(3):662–7. <https://doi.org/10.1093/humrep/dem429>.
 31. Adams RH, Eichmann A. Axon guidance molecules in vascular patterning. *Cold Spring Harb Perspect Biol*. 2010;2(5):a001875. <https://doi.org/10.1101/cshperspect.a001875>.
 32. Li N, Zhan X. Signaling pathway network alterations in human ovarian cancers identified with quantitative mitochondrial proteomics. *EPMA J*. 2019;10(2):153–72. <https://doi.org/10.1007/s13167-019-00170-5>.
 33. Garcia-Jerez A, Luengo A, Carracedo J, Ramirez-Chamond R, Rodriguez-Puyol D, Rodriguez-Puyol M et al. Effect of uraemia on endothelial cell damage is mediated by the integrin linked kinase pathway. *J Physiol*. 2015;593(3):601–18; discussion 18. <https://doi.org/10.1113/jphysiol.2014.283887>.
 34. Feletou M, Kohler R, Vanhoutte PM. Endothelium-derived vasoactive factors and hypertension: possible roles in pathogenesis and as treatment targets. *Curr Hypertens Rep*. 2010;12(4):267–75. <https://doi.org/10.1007/s11906-010-0118-2>.
 35. Kosaka K, Fujiwara H, Yoshioka S, Fujii S. Vascular endothelial growth factor production by circulating immune cells is elevated in ovarian hyperstimulation syndrome. *Hum Reprod*. 2007;22(6):1647–51. <https://doi.org/10.1093/humrep/dem042>.
 36. Adams RH, Eichmann A. Axon guidance molecules in vascular patterning. *Cold Spring Harb Perspect Biol*. 2010;2(5):a001875. <https://doi.org/10.1101/cshperspect.a001875>.
 37. Hatzirodos N, Irving-Rodgers HF, Hummitchsch K, Harland ML, Morris SE, Rodgers RJ. Transcriptome profiling of granulosa cells of bovine ovarian follicles during growth from small to large antral sizes. *BMC Genomics*. 2014;15:24. <https://doi.org/10.1186/1471-2164-15-24>.
 38. Baufeld A, Koczan D, Vanselow J. L-lactate induces specific genome wide alterations of gene expression in cultured bovine granulosa cells. *BMC Genomics*. 2019;20(1):273. <https://doi.org/10.1186/s12864-019-5657-6>.
 39. Baufeld A, Vanselow J. Lactate promotes specific differentiation in bovine granulosa cells depending on lactate uptake thus mimicking an early post-LH stage. *Reprod Biol Endocrinol*. 2018;16(1):15. <https://doi.org/10.1186/s12958-018-0332-3>.
 40. Zeilmaker GH, Verhamme CM. Lactate concentrations in pre-ovulatory follicles of pro-oestrous rats before and after onset of

- oocyte maturation. *Acta Endocrinol.* 1977;86(2):380–3. <https://doi.org/10.1530/acta.0.0860380>.
41. Xie Z, Chen WS, Yin Y, Chan EC, Terai K, Long LM, et al. Adrenomedullin surges are linked to acute episodes of the systemic capillary leak syndrome (Clarkson disease). *J Leukoc Biol.* 2018;103(4):749–59. <https://doi.org/10.1002/jlb.5a0817-324r>.
42. Grøndahl ML, Andersen CY, Bogstad J, Borgbo T, Boujida VH, Borup R. Specific genes are selectively expressed between cumulus and granulosa cells from individual human pre-ovulatory follicles. *Mol Hum Reprod.* 2012;18(12):572–84. <https://doi.org/10.1093/molehr/gas035>.
43. Poulsen LC, Bøtkjær J, Østrup O, Petersen KB, Yding Andersen C, Grøndahl ML, et al. Two waves of transcriptomic changes in periovulatory human granulosa cells. *Hum Reprod.* 2020; In press.
44. Mathur RS, Jenkins JM, Bansal AS. The possible role of the immune system in the aetiopathogenesis of ovarian hyperstimulation syndrome. *Hum Reprod.* 1997;12(12):2629–34. <https://doi.org/10.1093/humrep/12.12.2629>.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.