Title: Neuropeptide S Receptor 1 is a Novel Non-Hormonal Treatment Target in Endometriosis

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One Sentence Summary: Using genetic analyses in humans and rhesus macaques, followed by in-vitro cellular and in-vivo inhibition studies, we present evidence that NPSR1 is a novel non-hormonal drug target in endometriosis.

Abstract

Endometriosis is a common chronic inflammatory condition causing pelvic pain and infertility in women, with limited treatment options and a heritability of 50%. We aimed to leverage genetic analyses in two species with spontaneous endometriosis, humans and the rhesus macaque, to uncover novel treatment targets. We sequenced DNA from 32 human families contributing to a genetic linkage signal on chromosome 7p13-15 and observed significant over-representation of predicted deleterious low-frequency coding variants in *NPSR1* in cases (predominantly stage III/IV) vs. controls ($P=7.8 \times 10^{-4}$). Significant linkage to the region orthologous to human 7p13-15 was replicated in a pedigree of 849 rhesus macaques ($P=0.0095$). Targeted association analyses in 3194 surgically confirmed, unrelated cases and 7060 controls revealed that a common insertion/deletion variant, rs142885915, was significantly associated with stage-III/IV endometriosis ($P=5.2 \times 10^{-5}$; Odds Ratio=1.23 (95% CI=1.09-1.39)). Immunohistochemistry, qRT-PCR and flow cytometry experiments demonstrated that NPSR1 was expressed in glandular epithelium from eutopic/ectopic endometrium, and on monocytes in peritoneal fluid from cases and controls. The NPSR1 inhibitor SHA68R blocked NPSR1-mediated signaling, pro-inflammatory TNF- α release and monocyte chemotaxis *in-vitro* ($P<0.01$), and *in-vivo* led to a significant reduction of inflammatory cell infiltrate and abdominal pain ($P<0.05$) in a mouse model of peritoneal inflammation (zymosan-induced peritonitis) and a mouse model of

1 endometriosis. We conclude that the NPSR1/NPS system is a new, genetically validated, non-
2 hormonal target for the treatment of endometriosis with likely increased relevance to stage III/IV
3 disease.

5 **Introduction**

6 Endometriosis, the growth of endometrial-like tissue outside the uterus, is a common, chronic,
7 estrogen-dependent inflammatory condition affecting an estimated 5-10% of women (190
8 million globally) in their reproductive years (1). Lesions are primarily located on pelvic organs,
9 and the disease often causes intense pelvic pain exacerbated by menstruation, as well as reduced
10 fertility. The total annual societal cost of endometriosis-associated symptoms has been estimated
11 at US \$50bn in the USA alone, incurring direct healthcare costs similar to type 2 diabetes (2).

12 Endometriosis is most commonly classified surgically according to the revised American
13 Society of Reproductive Medicine (ASRM) stage I-IV classification system (3). However,
14 surgical stage correlates poorly with pelvic pain severity (4). The pain experienced as the
15 predominant symptom of endometriosis is likely caused by chronic localized inflammation (5),
16 even after lesions have been removed or destroyed (6), with peripheral and central nervous
17 sensitization contributing to the overall pathophysiology (7). Current treatment options are
18 limited to invasive surgical procedures to remove or destroy lesions, or hormonal treatments that
19 can have marked side-effects (8).

20 The causes of endometriosis remain largely elusive, but heritability has been estimated at around
21 50% (9). Here, we first use genetic analyses in human family- and population-based studies,
22 complemented by a study of a large pedigree of rhesus macaques (*Macaca mulatta*) with

spontaneous endometriosis, to identify novel associations with low-frequency (minor allele frequency (MAF)<0.05) and common variants in the neuropeptide S (NPS) receptor 1 (*NPSR1*); and subsequently conduct both *in-vitro* and *in-vivo* target validation and inhibition studies of *NPSR1* to demonstrate its promise as a novel treatment target for endometriosis.

Results

Low-frequency coding variants in *NPSR1* are associated with familial endometriosis

We previously showed significant linkage of chromosome 7p13-15 with endometriosis, likely attributable to low-frequency risk variants, in an analysis of 52 families each with three or more women surgically diagnosed with the disease (10). We conducted fine-mapping non-parametric linkage analysis and haplotype analysis of this region using 32 families that contributed to the linkage signal (see Methods) and narrowed the region of interest to a 5 Mb segment flanked by D7S683 and D7S510, centered around D7S2251. This chromosomal segment contained 28 genes (Figure 1 A).

We next sequenced this 5 Mb region in eight unrelated women from the eight families contributing most to the linkage signal. Of the 28 genes in the region, five harbored ≥ 1 non-synonymous, predicted deleterious coding variants (see Methods) in at least 50% of the sequenced families: *EEPD1*, *KIAA0895*, *AOAH*, *ELMO1* and *NPSR1*. We performed targeted sequencing of these five genes in the remaining 100 cases and five controls from the 32 families contributing positively to the 7p13-15 linkage signal (Logarithm of Odds, LOD, >0.1), and in an additional 100 unrelated population-based controls (for details, see Methods and Supplementary Methods). We found an over-representation of cases with ≥ 1 low-frequency variants (Minor

Allele Frequency (MAF) <0.05), non-synonymous coding variants in *NPSRI* compared to controls (Figure 1 B).

In total, three different missense variants in *NPSRI* were found in nine out of 32 families (Pedigrees in Supplementary Figure 1): 1) a novel variant c7_34698148 (p.Gly42Cys, coordinates Build 37) in a family with five affected sisters (the family contributing most to the linkage signal) where the variant was present in all affected sisters; 2) two low-frequency variants, rs34705969 (1000 Genomes Phase V3 (1000GV3): MAF=0.025, p.Cys197Phe) in four families, present in 6/12 genotyped cases, and 3) rs116825950 (1000GV3: MAF = 0.025, p.Gly358Asp) in another set of four families, present in 11/12 genotyped cases. Not counting the novel variant, based on the population-based MAF of the two known variants, we would have expected three of the 32 families to carry one of the two known single nucleotide polymorphisms (SNPs) (Supplementary Methods), but we found eight such families. The binomial probability of observing eight out of 32 families carrying at least one of these known variants on the basis of their population frequency is $P=7.8 \times 10^{-4}$, a significant over-representation. Of the 25 cases with known ASRM disease stage in the nine families, 18 (72%) had been diagnosed with stage III/IV and 7 (38%) with stage I/II.

We subsequently genotyped all three missense variants implicated in familial endometriosis, as well as 20 other coding variants in *NPSRI* present on the Illumina Infinium Exome array (Supplementary Methods) in 3155 endometriosis cases and 2332 population controls from two datasets as part of a genome-wide association study (GWAS) (11). One of the three familial variants (rs34705969) could not be genotyped due to primer design issues, and of the remaining 22 variants, only five with MAF <0.01 and two with MAF <0.10 were polymorphic (Table S1). Single SNP analysis of these low-frequency variants did not show significant

association with overall endometriosis, nor did burden analysis of the minor allele count across SNPs with overall endometriosis ($P=0.12$). However, aggregate analysis limited to cases with a known family history of endometriosis showed significant association ($P=0.04$).

Common variants of *NPSR1* are associated with endometriosis

The implicated variants in *NPSR1* were low-frequency coding variants. We next investigated whether common variants mapping to/near *NPSR1* and its ligand *NPS* were also associated with endometriosis, using a genome-wide association dataset of surgically confirmed cases (11). We analyzed all common variants ($MAF>0.01$) $\pm 500Kb$ of *NPSR1* from a published genome-wide association dataset of all endometriosis (3194 cases), stage I/II (1364 cases), stage III/IV (1686 cases) and familial stage III/IV (606 cases) vs. 7060 controls (see Methods). Using a Bonferroni-adjusted P-value threshold of $P<5.5\times 10^{-5}$ allowing for 903 independent tests (Supplementary Methods), we observed a significant association of rs142885915 in *NPSR1* (Indel chr7:34,855,387, $MAF = 0.11$) with stage III/IV endometriosis ($P=5.15 \times 10^{-5}$, $OR=1.23$, 95% $CI=1.09-1.39$; Figure 1 C), but not when including all endometriosis stages, or stage I/II disease alone (Supplementary Figures 2 A-C). Data from the GTex tissue expression database (12) suggests that the deletion (G- vs. GA) at rs142885915 is associated with reduced expression of *NPSR1* in cerebellum ($N=209$; normalised effect size=-0.58, $p=6.1\times 10^{-5}$), but with increased expression in the frontal cortex ($N=175$; normalised effect size=0.255, $p=0.09$; Supplementary Figure 3). Rs142885915 was not in linkage disequilibrium with the two low-frequency variants rs34705969 ($r^2=0.006$) and rs116825950 ($r^2=0.002$) found in the family-based sequencing analysis. No significant association with common variants in *NPS* was observed (Supplementary Figure 2 D-F).

***NPSR1* variants in rhesus macaques diagnosed with endometriosis**

Parallel to the human studies, we conducted genetic analyses in a large multi-generational pedigree of rhesus macaques with spontaneous endometriosis, in which we had previously demonstrated familial aggregation of disease (13). Using both human- and rhesus macaque-specific microsatellite markers (Tables S2, S3, also see Extended Methods), we genotyped 418 out of the 849 phenotyped animals in regions orthologous to the two significant linkage regions previously identified in humans: human chromosomes 10q26 (14) and 7p13-15 (10). We conducted simulation-based non-parametric linkage analysis, obtaining empirical significance estimates to allow for the complex pedigree structure (see Methods). While no significant linkage was found on the macaque ortholog of human chromosome 10 (minimum $P=0.5$, Supplementary Figure 4), we found significant linkage between D7S497 located on rhesus macaque chromosome 3 (ortholog of human chromosome 7) and endometriosis (minimum $P=0.0095$ (D7S497); Figure 1 D).

Given the strong signal in *NPSR1* under the human linkage peak, we conducted targeted sequencing of *NPSR1* in 62 rhesus macaque cases and 49 controls (Table S4). We identified ten missense variants. Three rare missense variants were seen at a $MAF < 0.05$ in cases and were not observed in controls. Three additional missense variants were more prevalent in cases than controls. In aggregate, 44/544 (8.09%) of alleles in rhesus macaque cases carried at least one of the six missense variants exclusive to or prevalent in the cases compared with 26/512 (5.08%) alleles in rhesus macaque controls. Sample size was insufficient to warrant statistical testing for genetic association with disease after adjusting for familial relationships.

Combined, these parallel genetic analyses in two species strongly suggested a role for *NPSR1* in the pathogenesis of endometriosis.

NPSR1 is expressed in endometrial tissues and on monocytes in peritoneal fluid

Given the probable role of endometrial biochemistry and pathophysiology as well as immune response in endometriosis pathogenesis (1), we tested for the presence of NPSR1 and its cognate ligand NPS in endometrial tissue, as well as on immune cells present in peritoneal fluid (PF). Eutopic endometrial tissue and endometriotic (ectopic) tissue sections from 13 endometriosis patients with different ASRM disease stages and at different stages of the menstrual cycle, and eutopic endometrium from seven controls with no evidence of endometriosis were stained with anti-NPSR1 and anti-NPS antibodies (Figure 2 A; Supplementary Figure 5, isotype stainings; Supplementary Figure 6). NPSR1 was expressed strongly in glandular epithelial cells in both eutopic and ectopic endometrium, showing darkly stained nuclei surrounded by lighter-stained cellular membranes. Conversely, NPS was expressed in the stroma but not in the glandular epithelium, while NPSR1 staining was sparse in the stroma. We did not find a perivascular expression pattern of either NPSR1 or NPS (Supplementary Figure 7). This suggests a paracrine signaling mechanism within the organ with ligand and receptor in close proximity, similar to what has been observed in the lung (15) and the gastrointestinal tract (16).

We measured the expression of *NPSR1* in 49 samples by qRT-PCR (Figure 2 B) and found that amounts were similar in eutopic endometrium from endometriosis patients and unaffected controls, as well as in endometriotic tissue (n=11-26 per group, P=0.20; ANOVA with Dunnett's multiple comparisons test); expression did not depend on the ASRM stage or

cycle phase (n=2-5 per group, P=0.22; Kruskal-Wallis test, with Dunn's multiple comparisons test).

We next investigated whether immune cells in PF expressed NPSR1, as previously reported for alveolar macrophages in asthmatic lungs (17), and whether expression corresponded to amounts of NPS secreted into the PF (Figure 3). Flow cytometric analysis of PF cells showed that the majority of hematopoietic cells (CD45⁺) expressed NPSR1 on their surface, with no significant difference in receptor expression between patients and controls (Figure 3 A; gating in Supplementary Figure 8). However, in analyses stratified on menstrual cycle phase, NPS amounts were significantly increased in the PF of patients vs. controls during the luteal phase and menstruation, but not during the proliferative phase (P=0.013 during luteal phase; P=0.024 during menstruation; 70 patients vs. 38 controls, Mann-Whitney U-test; Figure 3 B). We tested for association of the amount of NPS in PF, accounting for menstrual cycle phase: NPS amount was associated with disease status (P=0.0472; logistic regression adjusting for cycle phase, NPS amount as predictor and endometriosis status as outcome) but not cycle phase (P=0.7). We used mass cytometry (CyTOF) to discriminate between the NPSR1⁺ leukocyte populations of the PF and identified a population of infiltrating monocytes highly positive for NPSR1 in patients but not in controls (Figure 3 C, Tables S5, S6). No differences in NPSR1 expression by menstrual cycle phase were observed (Supplementary Figure 9).

Inhibition of NPSR1 abrogates pro-inflammatory signalling in monocytes *in vitro*

Monocytes have previously been described as expressing NPSR1 (17), which signals upon binding of NPS through the G α_q and G α_s subunits of the G protein coupled to the receptor, and subsequently through Ca²⁺ mobilisation and cAMP signalling, respectively (Figure 4 A(18)).

Downstream effects of NPS signalling include TNF- α release (19) and chemotactic behaviour towards NPS (20).

We used the small molecular inhibitor SHA 68 (3-Oxo-1,1-diphenyl-tetrahydro-oxazolo[3,4-a]pyrazine-7-carboxylic acid 4-fluoro-benzylamide, Figure 4 B (21)) to inhibit NPSR1 signalling in monocytes and evaluated the inhibitory effect on different stages of NPS/NPSR1 signal transduction *in vitro*, including receptor dimerisation, subsequent release of Ca²⁺ and cAMP second messengers, TNF- α release, and on chemotaxis of human monocytes from healthy controls to NPS. After confirming the R enantiomer as the inhibitory isomer (Figure 4 C), we used SHA 68R in all subsequent experiments.

The inhibitor showed a dose-dependent inhibition of NPSR1 receptor activation as determined by reduced beta-arrestin binding (Figure 4 D), showing an EC₅₀ for NPS of 88 nM (pEC₅₀ = 7.06) and an IC₅₀ of 86 nM (pK_A = 7.07), confirming previous studies (22, 23). We found both cAMP and Ca²⁺ signalling inhibited by SHA 68R in a dose dependent fashion (Figure 4 E-G). In the cAMP assay, the EC₅₀ of NPS was 90 nM (pEC₅₀ = 7.04), and the IC₅₀ of the inhibitor SHA 68R was 15.3 nM (pK_A = 7.8), also as expected (21). Ca²⁺ signalling was quantified via increase in fluorescence of calcium-bound Fura Red dye (24), with the peak produced proportional to the NPS dose given (Figure 4 F; Supplementary Figure 10). We observed an inhibition of NPS induced Ca²⁺ mobilisation by 0.16 μ M SHA 68R on primary human monocytes (P=0.0187, ANOVA with Dunnett's multiple comparisons test against NPS 100 nM).

One of the hallmarks of endometriosis is a chronic inflammation in the peritoneum, with prominent involvement of cytokines such as TNF- α . We therefore tested the ability of SHA 68R to inhibit TNF- α release following LPS and NPS stimulation of monocytic THP-1 cells and

found that SHA 68R decreased the amount of TNF- α significantly at a dose of 10 nM (P=0.0140; ANOVA with Dunnett's multiple comparisons test against SHA 68R 0 nM, Figure 4 H).

Lastly, we tested the effects of SHA 68R on NPS-mediated monocyte chemotaxis to determine the dose to be used to inhibit monocyte infiltration *in vivo* (Figure 4 I). We used 100 nM of NPS to induce chemotaxis in human monocytes and determined an IC₅₀ of 28 nM for SHA 68R (pK_A = 7.55) against NPS-induced chemotaxis.

Taken together, SHA 68R significantly inhibited NPSR1 signalling in a dose dependent manner, demonstrating its ability to inhibit an inflammatory response *in vitro*.

We now tested the pharmacokinetics of SHA 68R in C57BL/6 mice to determine whether an appropriate dose could be given to maintain unbound inhibitor over the time frame needed for *in-vivo* experiments, and whether an injection *i.p.* or *s.c.* would be favourable (Figure 4 J). We found that an injection of 40 mg/kg of SHA 68R (*s.c.*) delivered sufficient amounts to inhibit monocyte chemotaxis (28 nM) for up to four hours without any side effects.

Inhibition of NPSR1 reduces pain and inflammation in peritonitis and endometriosis models

Finally, we investigated the effect of NPSR1 inhibition *in vivo* in inflammatory models used in endometriosis research (Figure 5, Supplementary Figures 11 and 12). We used the ZIP model, which shares many characteristics with human peritoneal inflammation, most importantly the influx of F4/80^{low} 7/4⁺ monocytes and increased cytokine amounts (25). We measured the number of infiltrating monocytes in comparison to the F4/80⁺ 7/4⁻ resident macrophages (Figure 5 A, B, Supplementary Figure 11) and found SHA 68R effective in reducing inflammation at 40

mg/kg as evidenced by a reduction in the number of infiltrating monocytes (Figure 5 B, n=9–12 mice per group, ** P=0.0011, ANOVA; ** P=0.0096, test for linear trend 0, 10, 40 mg/kg SHA 68R). Blood plasma cytokine amounts of the inflammatory cytokines IL-4, IL-12p40 and IL-17 were significantly reduced (Figure 5 C; n=8-10 mice per group, IL-4 * P=0.0413, IL-12p40 ** P=0.0010, IL-17 * P=0.0409, 40 mg/kg vs control). Because not all mice produced data points within the detection limit, a 2-way ANOVA could not be done and we used a mixed-effects model analysis instead, with Holm-Sidak correction for multiple comparisons. We confirmed the anti-inflammatory effect of SHA 68R in an experimental model of endometriosis, involving the inoculation of 12 uterine tissue fragments derived from syngeneic estrous phase matched donor mice into the peritoneal cavity. Again, the amounts of inflammatory cytokines were decreased by SHA 68R (Figure 5 D, Supplementary Figure 12; n=8–11 mice per group, IL-1b * P=0.0228, IL-12p40 * P=0.0178). Lastly, to measure the effect of NPSR1 inhibition on abdominal pain as the main symptom of endometriosis, we used the dynamic weight bearing assay (26) (Figure 5 E, F) and found SHA 68R effective in reducing abdominal pain-related spontaneous behaviors in both the ZIP (n=10 mice per group, P=0.0315, unpaired t-test, 40 mg/kg SHA 68R vs control) and endometriosis inoculation models (n=8 mice per group, P=0.0263, unpaired t-test, 40 mg/kg SHA 68R vs control). In line with previous studies, the induction of pain-associated behavior became apparent in the control groups during day 2 (Figure 5 F), with SHA 68R significantly reducing pain-associated behavior at the time of the therapeutic window.

Discussion

Endometriosis represents a substantial global public health burden and unmet clinical need that is under-recognized (1), with annual direct healthcare costs similar to those of type 2 diabetes.

1 Indirect costs associated with impaired quality of life and work productivity due to symptoms
2 that include pelvic pain, dysuria, dyschezia, chronic fatigue, and infertility far exceed direct
3 healthcare costs. Treatment of endometriosis is limited to surgical removal of endometriotic
4 tissue or hormonal medication mainly aimed at estrogen suppression. Alternative treatments with
5 fewer side-effects are urgently required. Despite genetic studies suggesting that the pathogenesis
6 of ASRM stage I/II disease is genetically distinct from stage III/IV (27), no treatments targeted
7 towards specific disease subtypes have been developed.

8 Our study combined evidence from genetic analyses of endometriosis in two species,
9 humans and rhesus macaques, to identify a novel, non-hormonal target for therapeutic
10 intervention: The G-protein-coupled cell surface receptor NPSR1. Naturally occurring primate
11 genetic models of human disease have been described for human single-gene disorders (28, 29),
12 but to our knowledge this is the first example of parallel investigations in which both human and
13 non-human primate data were used in a synergistic manner to identify a genetically validated
14 therapeutic target. Following our previously reported significant linkage signal for endometriosis
15 on chromosome 7p13-15 (10), we employed a combination of fine-mapping, haplotyping, and
16 next-generation sequencing approaches in families contributing to the linkage signal, and
17 additional linkage and sequencing analysis in rhesus macaques with spontaneous disease. These
18 analyses uncovered significant association of low-frequency, missense coding variants in *NPSR1*
19 with familial, predominantly ASRM stage III/IV, endometriosis. Targeted analyses of large-scale
20 GWAS data for surgically confirmed cases (11) also highlighted significant association of
21 common non-coding *NPSR1* variants with stage III/IV endometriosis in the general population.

22 Genetic variants in *NPSR1* have been associated with a range of inflammatory and
23 neuropsychiatric phenotypes, including asthma (30, 31), allergy (32, 33), inflammatory bowel

1 disease (IBD) (16, 34), rheumatoid arthritis (35), panic disorders (36), psychological stress (37),
2 fear responses (38), and recurrent abdominal pain (39). The common insertion/deletion variant
3 associated with endometriosis in *NPSR1*, rs142885915, is intronic, has a frequency of 11.3% in
4 European ancestry populations, and is independent from common variants previously associated
5 with IBD, asthma and neuropsychiatric phenotypes (linkage disequilibrium between variants
6 $r^2 < 0.1$). GTex tissue expression data suggest that the effects of the deletion are complex and
7 likely tissue-dependent, with evidence for associated downregulation of *NPSR1* in cerebellum
8 and upregulation in the frontal cortex. Intriguingly, independent variants in *NPSR1* previously
9 associated with abdominal pain, rs2530566G (40) and inflammatory bowel disease, rs2530547
10 (41), show the exact same pattern in GTex (accessed January 2021) of association with reduced
11 *NPSR1* expression in cerebellum and increased expression in the frontal cortex. Both brain areas
12 - but the frontal cortex in particular - are activated in pain perception and anticipation (42). The
13 tissue-specific effects of this deletion following sustained inflammatory insult such as seen in
14 endometriosis require further functional work in the context of other *NPSR1* polymorphisms.
15 One of the low-frequency missense mutations observed to be associated with familial
16 endometriosis, rs34705969 (p.Cys197Phe), has previously been described as affecting *NPSR1*
17 signaling properties through structural alteration of the receptor resulting in a reduced ability to
18 induce either downstream gene expression changes or cAMP-mediated signaling upon NPS
19 stimulation (41). The effects of the other two missense variants rs116825950 (p.Gly358Asp) and
20 the novel variant C7_34698148 (p.Gly42Cys) on *NPSR1* structure or function are unknown.
21 Studying the effect of the identified missense *NPSR1* mutations/variants on *NPSR1* signaling by
22 CRISPR/Cas9-based mutation of *NPSR1* within relevant primary cells, e.g., monocytes and
23 epithelial endometrial cells, would be a worthwhile aim of future studies.

1 The genetic validation of the potential role of NPSR1 in endometriosis is important as it
2 greatly increases the likelihood for a drug target modulator to be approved for clinical use (43).

3 Of note, NPS is encoded on chromosome 10p26.2, the second significant linkage area reported
4 for endometriosis (44). Specifically, NPS is located 1.3 Mb outside the boundary of the highest
5 confidence LOD-1 support region underneath the linkage peak, but 192kb within the broader
6 region defined by Maximum LOD Score (MLS) > 1. Based on this positional evidence, future
7 studies may benefit from also investigating the role of rare NPS variants on the cell-specific
8 function of NPS in endometriosis. Following our genetic analyses, we demonstrated functional
9 effects of *NPSR1* on pro-inflammatory processes in both *in vitro* and *in vivo* experiments.
10 Limitations of our investigations include the relatively small number of patients studied in the *in-*
11 *vitro* assays, and the limitations inherent to rodent models for endometriosis and pelvic pain,
12 which will not mimic the full, complex physiological spectrum of human endometriosis.
13 Nevertheless, we identified a population of infiltrating monocytes highly positive for NPSR1 in
14 women with endometriosis but not controls and showed that inhibition of NPSR1 through a
15 selective inhibitor, SHA68 R, resulted in reduced migration of NPS-induced monocytes *in-vitro*.
16 We further demonstrated an anti-inflammatory effect and a reduction of abdominal pain-induced
17 behavior following SHA68R administration *in-vivo*, in both a ZIP mouse model (25) and an
18 inoculation model of endometriosis (45), with concomitant reduction in the number of
19 infiltrating monocytes and decreased blood cytokine amounts. The ZIP model features all stages
20 of inflammation including the resolution phase, allowing for the study of different immune cell
21 types involved at different stages, while the endometriosis model includes the presence of
22 endometrial adhesions within the peritoneal cavity, thus mimicking the presence of lesions.

1 The chronic inflammatory phenotype associated with endometriosis is likely maintained
2 by immune cells in PF (1). Inflammation and pain are thought to be triggered by the deposition
3 of endometrial cellular material within the peritoneum (5), although the pain mechanisms
4 underlying endometriosis are complex and can be of inflammatory as well as neuropathic nature
5 (1). Pivotal to pathology is an aberrant wound-healing response of monocytes and resident
6 macrophages to cellular debris (46). The increased amounts of NPS in endometriosis patients
7 during the secretory and menstrual phases of their cycle could augment this aberrant response by
8 increasing monocyte recruitment. Whether this response is different in ASRM stage I/II vs. stage
9 III/IV disease is unknown, but our genetic results appear to indicate that NPSR1/NPS related
10 monocyte response may be particularly relevant in stage III/IV disease.

11 NPSR1 expression on inflammatory cells, typically induced by inflammatory cytokines
12 such as TNF- α and interferon- γ (16), is upregulated during inflammation in asthma (30) and IBD
13 (34). Monocytes and macrophages isolated from mice in asthma models expressed NPSR1 after
14 antigen challenge (47), and stimulation of these macrophages with NPS led to pro-inflammatory
15 M1 polarization and increased migration. The NPS/NPSR1 system in turn upregulated the
16 expression of IL-8, one of the most prominent monocyte attractants and pro-inflammatory
17 cytokines (48), and a key cytokine in endometriosis inflammatory pathophysiology (1). Thus,
18 NPS-induced signaling through NPSR1 could serve as a point of departure for chronic
19 inflammation, with a perpetuated feedback loop through IL-8 dependent recruitment of
20 inflammatory monocytes, TNF- α induced upregulation of NPSR1 and further production of
21 TNF- α by the new arrivals. This raises the prospect of clinically targeting NPSR1 to interrupt the
22 developing chronic inflammation in endometriosis and alleviate pain as its main symptom. We
23 have shown in animal models that NPSR1 inhibition holds promise towards this goal.

1 We acknowledge that our study has some limitations. In endometriosis, we address a
2 complex human condition of unknown aetiology. Some of our investigations did not allow for
3 more detailed analyses than presented here, which would have been desirable. As mentioned, our
4 *in-vitro* studies did not include investigations of the functional effects of the *NPSR1* variants we
5 identified to be associated with endometriosis, a topic of interest for future studies. Secondly, we
6 focused on the inflammatory effects related to NPSR1/NPS, and did not investigate its anxiety-
7 related effects described in the literature, which might have additional bearing on the experience
8 of endometriosis-related pain symptoms. A final point is that mouse models inevitably vary in
9 the degree to which they approximate endometriosis in menstruating species. While mouse
10 models mimic elements of the endometriosis disease process, they cannot feature the full disease
11 spectrum, in particular the pathological nuances related to disease stages seen in women. Further
12 exploration of the effect of NPSR1/NPS inhibition on endometriosis-related inflammation and
13 pain symptoms in the rhesus macaque would be very desirable.

14 In summary, endometriosis is a common condition for which better targeted, non-
15 hormonal treatment options are urgently needed. Our results suggest the NPSR1/NPS system is a
16 novel, genetically validated, non-hormonal target with likely increased relevance to stage III/IV
17 endometriosis therapeutics.

Materials and Methods

Detailed methods are available in the Supplementary Materials and Methods.

Study design: The aim of this study was to 1) identify genetic target(s) from families with multiple cases of endometriosis and 2) explore the role of the candidate gene derived from these analyses, NPSR1, as a novel non-hormonal treatment target for the disease. Fine-mapping linkage, haplotype and sequencing analyses were conducted using 52 families with 3+ women with endometriosis from the UK and Australia, and a large pedigree of 849 rhesus macaques with spontaneous disease. No selection of families or animals within the pedigree was applied, all available genotype data was used. For subsequent *in vitro* NPSR1/NPS description and validation experiments we used tissue and peritoneal fluid samples collected according to WERF EPHeCT standardized protocols (50). We tested inhibition of NPSR1 signalling in monocytes from cases and healthy controls under identical experimental conditions; and *in vivo* inhibition of inflammation and pain read-outs in a peritonitis mouse model (25) a mouse model of endometriosis (26). Sample sizes were not predetermined through statistical methods. The investigators were not blinded to the experimental conditions during experiments and the analyses.

Fine-mapping of the 7p13-15 linkage region in humans: We previously identified significant linkage to chromosome 7 (D7S493-D7S502) using 23 polymorphic markers, based on 52 families with three or more affected women from the OXEGENE Study (10) (Ethics approval: MREC/99/5/11). We genotyped an additional 31 markers (Table S2) across the linkage region in the 32 families contributing positively to the linkage signal (LOD > 0.1). Linkage analyses and maximum likelihood haplotypes construction were conducted using Merlin (52).

Linkage analysis in rhesus macaques: Fourteen microsatellite markers (Table S3) across two regions orthologous to the human regions previously exhibiting significant linkage in human families (7p13-15 and 10q26 (10)) were genotyped (seven markers per region) in 418 rhesus macaques from a pedigree at the Wisconsin National Primate Research Center, including 135 females with spontaneous endometriosis (53). Simulation-based non-parametric linkage analyses were performed using SimWalk2 (54).

Sequencing in humans and rhesus macaques: A 5 Mb genomic fragment centered on D7S2251 (Figure 1 A) was sequenced in eight unrelated affected women from the eight families contributing most to the 7p13-15 linkage signal. A custom NimbleGen array was used to capture the region of interest, followed by Roche 454 GS-FLX next-generation sequencing. Five out of 28 genes were prioritized for further analysis as they harbored ≥ 1 non-synonymous, low-frequency coding variant in $\geq 50\%$ of sequenced women. The five genes were sequenced (paired-end) on Illumina HiSeq following custom NimbleGen capture in 100 remaining cases and five controls from the 32 families and 100 unrelated controls (see Extended Methods) (10). Sanger sequencing was used to analyse the rhesus macaque region orthologous to human *NPSR1* in 62 affected animals and 49 unaffected controls with sufficient quality DNA (Table S4, see also Extended Methods).

Tissue and peritoneal fluid samples: Eutopic and ectopic endometrium as well as peritoneal fluid (PF) were collected from women recruited in the ENDOX Study (49) (REC reference 09/H0604/58, South Central - Oxford B Research Ethics Committee) according to WERF EPHeCT standardized protocols (50).

Immunohistochemistry: Samples were collected in ice cold phosphate buffered saline (PBS), fixed in paraformaldehyde (4%, 1 h) and ethanol (100%, overnight) and embedded in

paraffin blocks. Serial sections (5 μ m) were used for hematoxylin/eosin (H&E) and antigen specific staining. Antibodies used were anti-NPSR1, 1:1500; anti-NPS, 1:1000 (Abcam, Cambridge, UK), an anti-rabbit secondary antibody and DAB visualization with hematoxylin counterstain.

qRT-PCR: RNA was extracted from snap-frozen tissue samples and 1 μ g of RNA per sample reversely transcribed. 100 ng of cDNA were used in a SYBR Green® real-time PCR assay. Primers and conditions were as published previously (41), with a melting curve run to ascertain specific amplification. Relative expression of *NPSR1* vs. *GAPDH* was calculated using the $\Delta\Delta$ ct-method (55).

Flow Cytometry: Pure PF samples were washed and stained (Supplemental Extended Methods) and incubated with anti-NPSR1-AF488 (polyclonal rabbit) or an isotype control (both Bioss Inc, USA) and anti-CD45 BV605 (BD Biosciences, UK) at a 1:320 dilution for 1 h on ice in the dark. Cells were washed twice in PBS/BSA (1%), resuspended in 100 μ L and measured in a Fortessa flow cytometer (Beckton Dickinson, Oxford, UK).

CyTOF: Purified antibodies (Table S5) were labelled with metal tags using Maxpar® Antibody Labelling Kits (DVS Sciences) as per manufacturer's instructions. Labelled antibodies were titrated to determine the working concentration. Details of the patient samples used for CyTOF analysis are given in Table S6.

ELISA: Neuropeptide S (NPS) in PF supernatants was measured by ELISA (CEA796Hu, Cloud Clone Corp., USA) according to the manufacturer's instructions.

SHA 68R: The inhibitor compound was synthesized as previously described (51).

1 **Monocytes:** Primary human monocytes were isolated from blood samples from healthy
2 donors using Ficoll-Paque PLUS/Percoll (GE Healthcare Life Sciences, USA) centrifugation
3 according to the manufacturer's instructions. (Detailed method in Supplementary Appendix)

4 **Mouse experiments:** All experiments were performed at Bayer AG in accordance with
5 local policies and directives (Landesamt für Gesundheit und Soziales Berlin, Germany), and at
6 Oxford in accordance with the Animals (Scientific Procedures) Act 1986 Amendment
7 Regulations 2012 (ASPA 2012), UK.

8 **Zymosan induced peritonitis (ZIP) model:** The ZIP model was performed as
9 previously described (25). Female C57BL/6 mice (8–12 weeks old) were injected
10 subcutaneously with SHA 68R (40 mg/kg in *HP- β -cyclodextrin*, 10%, 50 μ L) or vehicle. One
11 hour later, zymosan was administered (0.1 mg in 200 μ L PBS intraperitoneally). After 4 h, tail
12 vein blood samples were collected under terminal anesthesia and the mice sacrificed, the
13 abdominal skin opened, and the peritoneum washed with 3 mL of ice-cold PBS with 5 mM
14 ethylenediaminetetraacetic acid (EDTA) to obtain peritoneal exudate cells (PEC). PEC were
15 counted and stained using anti-CD45 APC-Cy7 (530-F11, Biolegend, UK), anti-F4/80 BV421
16 (BM8, Biolegend) and anti-7/4 PE (AbD Serotech, UK). The myeloid cell populations were
17 gated for CD45 expression and plotted as F4/80 vs 7/4 in FlowJo (V10, Tree Star, Inc., USA).

18 **Endometriosis model:** Female BALB/c mice (8–12 weeks old) were injected
19 intraperitoneally with pieces of endometrium from syngeneic donor mice (8–10 pieces per
20 mouse). These pieces were recovered as lesions from the peritoneum two days later. The
21 inhibitor SHA 68R was given twice daily at 40 mg/kg *s.c.* on the day of inoculation and
22 throughout the experiment. PEC and blood were harvested as described above. The dynamic
23 weight bearing test (DWB) was done as described earlier (26).

Statistics *in vitro* and *in vivo* experiments: Unless stated otherwise, the non-parametric Kruskal-Wallis test with Dunn's test for multiple comparisons was used to compare mean values within groups; a $p < 0.05$ was deemed significant. Because of additional or missing data points, cytokine data were analyzed using a mixed-effects model instead, with the Holm-Sidak correction for multiple comparisons. Calculations were performed in Prism V8.1.1 (GraphPad Software, Inc.) and SPSS V23 (IBM Corp.).

1 **Supplementary Materials**

2 **Extended Materials and Methods**

3 **Supplementary Figures**

4 Supplementary Figure 1: Pedigrees of the nine families that carry the three identified missense
5 variants.

6 Supplementary Figure 2: Regional association plots of common variants in NPSR1 $\pm$ 500Kb and
7 NPS $\pm$ 500Kb with endometriosis.

8 Supplementary Figure 3: NPSR1 rs142885915 is an expression quantitative trait locus (eQTL) in
9 tissues analysed in the Genotype-Tissue Expression (GTEx) project.

10 Supplementary Figure 4: Linkage analysis of the region orthologous to human chromosome
11 10q26, chromosome 9 in the Rhesus macaque, using SimWalk2.

12 Supplementary Figure 5: Isotype control staining on eutopic and ectopic endometrial sample
13 sections.

14 Supplementary Figure 6: Immunohistochemistry images of NPS and NPSR1 stainings in
15 secretory phase endometrial tissue.

16 Supplementary Figure 7: NPS and NPSR1 are not expressed in a perivascular pattern.

17 Supplementary Figure 8: Gating strategy for evaluation of peritoneal fluid cells.

18 Supplementary Figure 9: The menstrual cycle phase does not influence the percentage of
19 NPSR1⁺ cells in peritoneal fluid immune cell populations in endometriosis patients.

20 Supplementary Figure 10: Ca²⁺ release measurements using Fura Red.

Supplementary Figure 11: The inhibition of NPSR1 reduces peritoneal inflammation in vivo in C57BL/6 mice.

Supplementary Figure 12: The inhibition of NPSR1 reduces inflammatory cytokines and pain in a mouse model of endometriosis.

Supplementary Tables

Table S1: Results of the aggregate analysis of 7 polymorphic coding variants in NPSR1 in 3155 human cases and 2332 controls.

Table S2: The 54 polymorphic markers used in fine-mapping of the 7p13-15 linkage region in humans.

Table S3: The 14 markers used for rhesus macaque linkage analyses.

Table S4: Targeted sequencing results of 10 SNPs in NPSR1 gene in 62 rhesus macaque cases and 49 controls.

Table S5: Markers used for the separation of peritoneal cell populations by viSNE algorithm.

Table S6: Peritoneal Fluid samples analysed by CyTOF.

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2 employees and shareholders of Bayer AG Pharmaceuticals.
- 3 **Data and materials availability:** All data will be made publicly available upon publication.
4

Figure 1: Evidence for rare and common genetic variants associated with *NPSR1* from the 7p14.2 region in Humans and Rhesus macaque. (A) Physical location plot (HG Build 19) of the original linkage evidence from 52 human families with 3+ endometriosis cases (*10*); additional fine-mapping linkage analysis in 32 families (yellow line); and linkage analyses in the rhesus macaque pedigree (green line). Each line represents the physical boundaries of

1 endometriosis linkage regions defined by LOD-1. The 5 Mb area selected for sequencing (red
2 line) is shown flanked by D7S683 – D7S510, centered around D7S2251. **(B)** Number of non-
3 coding, predicted deleterious, rare variants in 5 genes among 100 familial cases (orange) and 105
4 familial and population-based controls (blue). **(C)** Regional Locuszoom association plot for
5 *NPSRI* on chromosome 7p14.2 region for stage III/IV endometriosis (1686 cases vs. 7060
6 controls). Y-axis shows $-\log_{10}(\text{P-value})$ of association, and the x-axis genomic location (HG
7 build 19). The top associated SNP is coloured purple; other SNPs are coloured according to the
8 strength of LD with the top SNP (r^2 in the European 1000 Genomes V3 reference dataset; NB the
9 top associated indel, marked in grey, was not included given lack of LD data on non-biallelic
10 variants in the 1000GV3 reference dataset). **(D)** Significant linkage of endometriosis to the
11 region orthologous to human 7p14.2 in an extended pedigree of 849 rhesus macaques containing
12 135 affecteds (D7S497: $P=0.0095$).
13

Figure 2: NPSR1 and its ligand NPS are expressed in the endometrium. (A) Tissue samples of proliferative phase endometrium from patients with minimal/mild (stages I/II) or moderate/severe (stages III/IV) endometriosis were stained with anti-NPSR1 and anti-NPS antibodies, respectively (DAB, brown colour). Endometrium from disease-free controls was stained along with the patient samples. The lining of endometriotic glands (glandular epithelium) stained positive for NPSR1 irrespective of disease status and location of the endometrium (eutopic/ectopic), while the interstitium stained positive for NPS. (Magnification 200x, scale bar, 50 µm, representative images shown.) (B) Quantitative real-time PCR was done on eutopic and ectopic endometrium from patients and non-endometriotic controls. The expression of *NPSR1* was normalised to *GAPDH* using the $2^{\Delta\Delta_{ct}}$ -method and tested adjusted for cycle phase (E – eutopic, X – ectopic tissue). See Supplementary Figure 6 for staining of secretory phase endometrium.

Figure 3: NPS is elevated and NPSR1 prominently expressed on monocytes in peritoneal fluid from endometriosis patients. (A) Hematopoietic CD45⁺ cells from peritoneal fluid (PF) of endometriosis patients and non-endometriotic controls were stained with anti-NPSR1 (dark shaded peak) vs isotype controls (light) and measured by flow cytometry. NPSR1 is widely expressed on hematopoietic cells within peritoneal fluid in both controls and endometriosis (n=3

per group). **(B)** The amount of NPS in the PF of patients and controls was measured by ELISA. Samples were grouped by the menstrual cycle phase to account for variations caused by this. Compared to controls, NPS is significantly increased in the peritoneal fluid of endometriosis patients during the luteal phase ($P=0.013$; $n=19$, control and $n=32$, endometriosis) and menstruation ($P=0.024$; $n=4$, control and $n=10$, endometriosis; both Mann-Whitney U-Test) but not during the follicular phase ($n=15$, control and $n=28$, endometriosis). **(C)** Peritoneal fluid of 28 patients and 10 controls was analysed using cytometry by time-of-flight (CyTOF) and a panel of leukocyte markers (Table S5). The analysis of samples concatenated by group using the visual stochastic neighbour embedding (viSNE) algorithm reveals a population highly positive for NPSR1 within endometriosis patients but not in controls. Back-gating of the population shows the NPSR1⁺ cells to be CD11b⁺ inflammatory monocytes (arrows).

Figure 4: The specific inhibitor of NPSR1, SHA 68R reduces NPSR1-mediated inflammatory signalling in a dose-dependent manner. (A) Upon binding of its ligand NPS, NPSR1 signals downstream through the G protein subunits $G\alpha_q$ and $G\alpha_s$, with intracellular calcium (Ca^{2+}) and cAMP (18, 56). Downstream effects are TNF- α release (16) and chemotaxis (20). (B) Structure of the R and S enantiomers of SHA 68 (51). (C) Compared to the racemic mixture and the S enantiomer, the R enantiomer of SHA 68 has a stronger inhibitory effect on NPSR1 signalling in the β -arrestin assay (n=2 wells per dose). (D) The half-maximal inhibitory dosis (IC_{50}) of SHA 68R against NPS activation of NPSR1 was determined as 86.4 nM in the β -arrestin assay (NPS, 100 nM; n=4 wells per dose, 7 technical replicates). (E) Dose dependent inhibition of cAMP signalling; IC_{50} , 0.5 μ M (NPS, 100 nM; n=2 wells per dose). (F) Dose response curve of primary human monocytes loaded with Fura dye to indicate the intracellular release of calcium ions (Ca^{2+}) and stimulated with increasing doses of NPS. The release of Ca^{2+} ions leads to a peak in fluorescence (see Suppl Fig 6); the area under curve (AUC) is quantified as an indicator of signalling strength. NPS induces intracellular Ca^{2+} release in a dose-dependent manner, EC_{50} = 46.32 nM (n=8 wells per dose). (G) SHA 68R inhibits NPSR1 mediated Ca^{2+} signalling in a dose dependent manner. Primary human monocytes were pre-incubated with SHA 68R for 30 min and used in the Fura Red Ca^{2+} assay (* $P=0.0187$; *** $P=0.0002$; **** $P<0.0001$; n=2-14 per dose, 4 biological replicates; ANOVA with Dunnett's multiple comparisons test against NPS 100 nM). (H) SHA 68R inhibits the release of TNF- α in a dose dependent manner. THP-1 cells were pre-incubated with SHA 68R for 30 min at the doses indicated. Cells were stimulated with LPS and NPS, or with LPS alone. The amount of TNF- α released after 6 h was measured by ELISA (n=6 per dose, 3 biological replicates, SHA 68R 1

1 μ M, ** P=0.0013; SHA 68R 10 nM, * P=0.0331; SHA 68R 0.1 nM, * P=0.0140; ANOVA with
2 Dunnett's multiple comparisons test against SHA 68R 0 nM). N.B., stimulation with NPS alone
3 did not lead to significant TNF- α release. (I) SHA 68R inhibits NPS induced migration of human
4 monocytes in a dose dependent manner. Primary human monocytes were pre-incubated for 30
5 min with the indicated dose of SHA 68R and used in a chemotaxis assay against NPS over 2 h.
6 (n=4-6 wells per dose, 6 biological replicates; *** P<0.001 unpaired t-test SFM vs NPS 100 nM;
7 * P<0.05, ** p<0.01, ANOVA with Dunnett's post-test against NPS 100 nM; SFM, serum free
8 medium; ANOVA with analysis for trend within SHA 68R treatment groups, P=0.0017; IC₅₀,
9 28.31 nM). (J) Exposure testing of SHA 68R for *in vivo* experiments. C57BL/6 mice (n = 3 per
10 group) were injected *i.p.* or *s.c.* with the inhibitor SHA 68R (40 mg/kg) and blood samples were
11 taken at intervals to determine the amount of unbound SHA 68R in circulation. Data are
12 presented as means $\pm$ SEM.

Figure 5: The inhibition of NPSR1 reduces peritoneal inflammation and abdominal pain *in vivo*. The efficacy of NPSR1 inhibition *in vivo* was tested in mouse models. (A) C57BL/6 mice were injected subcutaneously (*s.c.*) with SHA 68R or vehicle one hour before zymosan was injected intraperitoneally (*i.p.*) to induce inflammation. An injection with PBS instead of zymosan was done to document the baseline distribution of cells within the peritoneum. Four hours later, the mice were sacrificed, and the peritoneal exudate was harvested. Cells were analysed by flow cytometry using markers for resident macrophages (F4/80) vs infiltrating monocytes (7/4). Plots shown are representative (n=9–12 mice per group). (B) Quantification of the F4/80-7/4⁺ inflammatory monocytes in the peritoneum. SHA 68R inhibited the infiltration of inflammatory cells in a dose-dependent manner (doses in mg/kg; n=9–12 mice per group, ** P=0.0011, ANOVA; ** P=0.0096, test for linear trend 0, 10, 40 mg/kg SHA 68R). (C) The analysis of cytokines in plasma shows a significant decrease in inflammatory cytokines after inhibition of NPSR1 signalling by SHA 68R in the ZIP model (n=8–10 mice per group; data

1 normalised to vehicle-only treatment, log2 up-/downregulation; IL-4 * P=0.0413, IL-12p40 **
2 P=0.0010, IL-17 * P=0.0409, 40 mg/kg vs control, mixed-effects model analysis with Holm-
3 Sidak correction for multiple comparisons). (D) The dose of 40 mg/kg of SHA 68R was used in
4 the endometriosis model in BALB/c mice and showed significant reductions of inflammatory
5 cytokines (n=8–11 mice per group; data normalised to vehicle-only treatment, log2 up-
6 /downregulation; IL-1b * P=0.0228, IL-12p40 * P=0.0178, mixed-effects model analysis with
7 Holm-Sidak correction for multiple comparisons). (E) The ratio of weight borne on the front vs
8 rear paws was measured as an indicator of abdominal pain 4 h after zymosan challenge and SHA
9 68R treatment (Veh, vehicle; Inh, 40 mg/kg SHA 68R *s.c.*). The dashed line indicates the
10 situation in PBS-injected mice. NPSR1 inhibition significantly decreased abdominal pain (n=10
11 mice per group, * P=0.0315, unpaired t-test). (F) In the BALB/c endometriosis model, dynamic
12 weight shifting was recorded 24 h and 48 h after inoculation, when pain manifests in this model
13 (n=8–10 mice per group, * P=0.0263, unpaired t-test). NPSR1 inhibition significantly reduced
14 abdominal pain in the endometriosis model.

Supplementary Materials

Extended Materials and Methods

Fine-mapping of 7p13-15 linkage regions in Humans

We previously identified significant linkage to chromosome 7 (7p13-15), based on an analysis of 52 families with 3 or more endometriosis cases from the OXEGENE study (10) (MREC/99/5/11). We genotyped an additional 31 markers across the linkage region (between D7S493 and D7S502), totaling 54 markers (Table S2), in 32 out of 52 Oxford families contributing positively to the linkage signal ($\text{LOD} > 0.1$). Linkage analyses and the maximum likelihood haplotypes were constructed using Merlin (52).

Linkage analysis in Rhesus macaque

We previously established a multigenerational pedigree of 1,831 rhesus macaques resident in a colony at the Wisconsin National Primate Research Center (WNPRC), University of Wisconsin (Madison), containing 155 affected animals with spontaneous endometriosis (13). Animals that were affected with endometriosis and/or of for which biological samples were available for DNA extraction were treated as informative. To maximise power, the pedigree was trimmed by removing animals for which no biological material was available and did not provide any connections between informative animals, using previously published computational methods (57). This process produced one pedigree containing 849 animals including 135 affected animals. Genomic DNA of 418 out of the 849 animals were extracted from blood, frozen tissue, or paraffin-embedded sections. Fourteen microsatellite markers across the two orthologous regions to human significant linkage regions (7p13-15 and 10q26), identified previously in our whole

genome linkage analyses, were selected and genotyped in the 418 animals (7 for each region; Table S3). Simulation-based non-parametric linkage analyses were performed using Simwalk2 (54), obtaining empirical significance estimates to allow for the complex pedigree structure.

Sequencing analyses in humans

A 5 Mb genomic fragment was selected for sequencing, centered on D7S2251 (chr7: 34.1 Mb - 39.2 Mb, hg19), covering the human and rhesus macaque linkage regions (Fig 1 A), in 8 unrelated affected women from the 8 human families contributing most to the linkage signal. At the Baylor Human Genome Sequencing Center (HGSC), genomic DNA of the target region was pulled down and enriched using a NimbleGen capture array with 5094 probes across the region. The collected DNA was subjected to pyro-sequencing on the Genome Sequencer FLX instrument using the Titanium series chemistry (454 Inc.). On average, approximately 162.1 Mb sequence was generated for each sample and the sequence depth was at least 20-fold (ranging from 20.0 to 33.8). Sequence coverage of the target region varied from 81.4% to 90.3%, with an average of 86.8%. A variant was identified and reported where (1) at least 3 non-duplicate reads differed either from the reference sequence or the other reads aligned at the specific position, (2) among the reads that show the difference, at least one read aligned in the forward direction and at least one aligned in the reverse direction, (3) the reads had at least 5 bases on both sides of the difference, (4) the reads had few isolated sequence differences in the read.

Prioritisation of genes for subsequent targeted sequencing was performed as follows. The original linkage analysis had suggested the causal variant(s) underlying the linkage peak might be rare in general population but with high frequency in the families contributing to the linkage signal. We prioritized variants identified from sequencing if they were (1) novel or with minor allele frequency (MAF) less than 0.05 based on data from HapMap and 1000Genomes and (2)

1 either non-synonymous, or located in predicted regulatory elements as reported in the Ensembl
2 functional genomics database (58), the FirstEF (first exon finder) in UCSC database (59) and the
3 DBTSS (database of transcription start sites) (60). An additional criterion was evolutionary
4 conservation based on two tracks from UCSC browser: 7×regPotential > 0.1 and phyloP 44-
5 way>0 (NB. 7×regPotential was computed from alignments of 7 vertebrates, and phyloP 44-way
6 was computed from multiple alignments of 44 vertebrates). Out of the 28 known genes in this
7 region, five (i.e. *NPSR1*, *EEPD1*, *KIAA0895*, *AOAH*, *ELMO1*) carried at least one above selected
8 variant in at least 4 out of the 8 families. These five genes were prioritized for further targeted
9 sequencing in the remaining 100 cases and 5 controls from the 32 families and an additional 100
10 unrelated controls randomly selected from the OXEGENE study. This second round of targeted
11 sequencing was also conducted at the HGSC. Sequence capture (enrichment) was performed
12 using custom probe sets and NimbleGen SeqCap EZ Developer Reagents. Illumina paired-end
13 sequencing libraries were prepared according to the manufacturer's recommendations and the
14 libraries sequenced on Illumina instruments (version Illumina 3.0).

15 We calculated the probability of observing the two known missense variants, rs34705969
16 and rs116825950 in a total of 8/32 families as follows. Each variant had a population-based
17 MAF of 0.025 (1000GV3). The probability of being a carrier in the general population for one
18 variant, assuming Hardy-Weinberg equilibrium of the variant in the population, is therefore
19 $0.025^2 + 2 \times 0.025 \times 0.975 = 0.049$. The probability of being a carrier of either variant is $2 \times 0.049 =$
20 0.09875 . The binomial probability of 8 out of 32 families containing a carrier of either variant is
21 $P = 7.8 \times 10^{-4}$.

Association analyses of rare and common *NPSR1* variants

The three missense variants implicated in familial endometriosis, along with 20 variants ($r^2 < 0.2$) in *NPSR1* present on the Illumina Infinium Exome array, were prioritized for genotyping in Oxford and Australian (QIMR Berghofer Medical Research Institute) datasets including 3155 surgically confirmed endometriosis cases and 2332 population controls. Genotyping was conducted at the QIMR Berghofer Medical Research Institute. Multiplex assays were designed using the Sequenom MassARRAY Assay Design software (version 3.0). SNPs were typed using Sequenom iPLEXTM chemistry on a MALDI-TOF Mass Spectrometer. The 2.5 μ L PCR reactions were performed in standard 384-well plates using 10 ng genomic DNA, 0.5 unit of Taq polymerase (Qiagen, Valencia, CA), 500 μ mol of each dNTP, and 100 nmol of each PCR primers. Standard PCR thermal cycling conditions and post-PCR extension reactions were carried out as described previously (61). The iPLEX reaction products were desalted by diluting samples with 15 mL of water and adding 3 mL of resin. The products were spotted on a SpectroChip (Sequenom Inc), and data were processed and analysed in a Compact Mass Spectrometer by MassARRAY Workstation (version 3.4) software (Sequenom Inc.). Out of the 20 variants genotyped, 7 were polymorphic (Table S1). Association with endometriosis was tested for each SNP, and in aggregate counting the minor alleles across all SNPs in cases vs. controls, using Chi-square and Fisher's exact tests.

In addition, a look-up of association with common variants in *NPSR1* was conducted in a published genome-wide association study involving the Oxford and Australian surgically confirmed datasets (11), re-imputed to the 1000G Phase V3 (1000GV3) reference panel, for: overall endometriosis (3,194 cases), stage I/II (1,364 cases), stage III/IV (1,686 cases) and familial stage III/IV (606 cases) vs. 7,060 controls. Taking the number of single nucleotide

polymorphisms (SNPs) tested and the regional linkage disequilibrium (LD) structure into account, the analyses involved approximately 903 tests for the *NPSR1* and *NPS* regions, resulting in a Bonferroni-corrected p-significance threshold of $p < 5.5 \times 10^{-5}$.

Sequencing analyses in rhesus macaques

In parallel with the second-round capture sequencing of human samples described above, we also performed targeted capture sequencing of the homologous regions in 23 rhesus macaques (13 macaques affected by endometriosis and 10 unaffected controls). Total genomic DNA was isolated at the Wisconsin NPRC using standard methods and shipped to the Baylor genome center. Genomic DNA fragments were selectively enriched using the same NimbleGen custom target-capture probes used for the studies of the five genes targeted in the second round of human sequencing. We again followed manufacturer's recommended procedures. Illumina paired-end sequencing libraries were constructed using standard manufacturer protocols, and sequencing performed on Illumina 3.0 instruments.

Expanded analysis of rhesus macaque sequence variation

Building on the sequencing and genotyping results for humans, we investigated genetic variation in *NPSR1* across 111 rhesus macaques, 62 affected with endometriosis and 49 unaffected controls (Table S4). We amplified exons from *NPSR1* using standard PCR methods, followed by Sanger chain termination sequencing on ABI 3730 instruments to genotype coding sequence polymorphisms in this sample set. Not every genotype was scorable in every animal. The average number of individuals scored across genotypes was 92.7 (minimum: 52; maximum: 104).

Samples for the experimental investigations

Eutopic and ectopic endometrium as well as peritoneal fluid (PF) were collected from women recruited in the ENDOX study, a prospective study of women aged 18-50 undergoing a laparoscopy for symptoms suggestive of endometriosis at the Endometriosis CaRe Centre, Oxford. Women who were pregnant, breastfeeding, or diagnosed with malignancy were excluded. Ethics approval for ENDOX was obtained from the NRES South Central-Oxford Research Ethics Committee (09/H0604/58). The study was approved by the Oxford University Hospitals Foundation Trust.

Immunohistochemistry

Samples were collected in ice cold PBS, fixed in paraformaldehyde (4%, 1 hour) and ethanol (100%, overnight) and embedded in paraffin blocks for cutting. Serial sections of 5 μ m thickness were used for haematoxylin/eosin (H&E) and antigen specific stainings. Antibodies used were anti-NPSR1, 1:1500; anti-NPS, 1:1000 (both Abcam, Cambridge, UK), with an anti-rabbit secondary antibody and DAB visualisation with hematoxylin counterstain. For perivascular staining, CD31, 1:800, and anti- α -smooth muscle actin (aSMA), 1:1000, were used (both Abcam, Cambridge, UK).

qRT-PCR

RNA was extracted from snap-frozen tissue samples and 1 μ g of RNA per sample reversely transcribed. 100 ng of cDNA were used in a SYBR Green® real-time PCR assay. The primers and conditions were used as published before (41), with a high resolution melting curve run to ascertain specific amplification. Relative expression of NPSR1 versus GAPDH was calculated using the $\Delta\Delta$ ct-method (55).

Primers: NPSR1 fw - CCCCTCATCTACTGTGTCTTCA; NPSR1 rv -
TCTCTCCCGGAACGTCATTCT; GAPDH fw - CCACATCGCTCAGACACCAT; GAPDH rv
- GCGCCCAATACGACCAAAT.

Flow Cytometry

Pure peritoneal fluid (PF) samples were washed twice in PBS/BSA (1%) and resuspended at a concentration of 10^6 cells/mL, blocked with human serum (10%) for 20 minutes and stained using the AquaStain live/dead stain at a 1:1000 dilution for 15 minutes. Samples were incubated with anti-NPSR1-AF488 (polyclonal rabbit) or an isotype control (both Bioss Inc, USA) and anti-CD45 BV605 (BD Biosciences, UK) at a 1:320 dilution for 1 hour on ice in the dark. Cells were washed twice in PBS/BSA (1%), resuspended in 100 μ L and measured in a Fortessa flow cytometer (Beckton Dickinson, Oxford, UK).

CyTOF

Pre-conjugated antibodies were purchased from DVS Sciences, purified antibodies from BioLegend, R&D Systems or Abcam (listed in Table S5). Purified antibodies were labelled with metal tags using Maxpar® Antibody Labeling Kits (DVS Sciences) as per manufacturer's instructions. Labelled antibodies were titrated to determine the working concentration. Details of the patient samples used for CyTOF analysis are given in Table S6.

ELISA

NPS in PF supernatants was measured by ELISA (CEA796Hu, Cloud Clone Corp., USA) according to the manufacturer's instructions. Results were analysed in Prism (V6, Graph Pad, Inc., USA).

SHA 68R

The inhibitor compound was synthesised as previously described (21, 51, 62) by Bayer AG, Berlin, Germany. The compound was dissolved in DMSO at 100 mM. Aliquots were frozen at -20°C until use.

Monocytes

Primary human monocytes were isolated from blood samples from healthy donors using Ficoll-Paque PLUS/Percoll (GE Healthcare Life Sciences, USA) centrifugation according to the manufacturer's instructions. Briefly, the blood volume was adjusted with PBS to 40 mL and divided into four tubes a 10 mL. These in turn were diluted with PBS to 25 mL volume. Each 25 mL volume of blood was then underlaid with 15 mL Ficoll-Paque PLUS and centrifuged at 800 g for 25 minutes with low acceleration and no brake. The resulting PBMC rings were harvested and washed in PBS three times before the lymphocytes were pelleted separately from the monocytes by centrifuging over a Percoll (GE Healthcare Life Sciences, USA) gradient (46% in RPMI) at 800 g for 25 minutes. The resulting ring of monocytes was harvested, washed in PBS and frozen at -80°C for further use.

Animals

Mice were acclimatized for one week and maintained in a 12h:12h light:dark cycle with access to food and water *ad libitum*. All experiments were performed in accordance with local policies and directives (LAGeSo (Landesamt für Gesundheit und Soziales Berlin, Germany, and Animals (Scientific Procedures) Act 1986 Amendment Regulations 2012 (ASPA 2012), UK).

Zymosan induced peritonitis (ZIP) model

The ZIP model was performed as described earlier (25). Female C57BL/6 mice (8 – 12 weeks old) were injected *s.c.* with SHA 68R (in HP- β -cyclodextrin, 10%, 50 μ L). Controls received vehicle only. One hour later, zymosan was administered (0.1 mg in 200 μ L PBS *i.p.*) before the mice were sacrificed 4 hours later. The skin above the abdomen was opened and the peritoneum washed with 3 mL of ice-cold PBS/5 mM EDTA to obtain peritoneal exudate cells (PEC). Blood samples were taken from the vena cava before sacrifice. PEC were counted and characterised using anti-CD45 APC-Cy7 (530-F11, Biolegend, UK), anti-F4/80 BV421 (BM8, Biolegend) and anti-7/4 PE (AbD Serotech, UK). The myeloid cell populations were gated for CD45 expression and plotted as F4/80 vs 7/4 in FlowJo (V10, Tree Star, Inc., USA).

Endometriosis model

BALB/c female mice 8-10 weeks old and weighing approx. 20 g (Janvier Labs) were used in the current study. After oestrus phase assessment, uteri from donor animals were collected and uterine punches were performed (2 mm diameter). Intraperitoneal injections of uterine biopsies in sterile PBS 37°C solution (12 uterine punches/recipient animal) were performed on recipient animals using 18G needles (Sterican). Animals were treated twice daily with SHA 68R dissolved in HP- β -cyclodextrin (10%) subcutaneously, starting 2h before inoculation. The inoculated pieces were recovered as lesions from the peritoneum two days later when the mice were sacrificed. Controls received vehicle only. The PEC and blood were harvested as described above.

Dynamic Weight Bearing (DWB) Assay

The dynamic weight bearing assay (DWB) was done one and two days after inoculation as described earlier (26). The automated DWB device (Bioseb, Boulogne, France) consists of a Plexiglas enclosure, a floor sensor consisting of receptors detecting variation in pressure, and a camera placed above the enclosure in order to validate the animal position during the analysis. DWB recording took place 4 h after zymosan injection (i.p., as above). The mice were able to move freely in the enclosure for 10 min, consisting of a habituation phase of 5 min followed by a recording phase of 5 min, in which weight distribution and positioning were recorded. We analysed recordings spanning the entire time period using the DWB software (v1.4.1.28). A zone was considered valid if the following conditions were met: ≥ 0.8 g on one receptor with a minimum of two adjacent receptors recording ≥ 1.0 g. A time segment was considered valid if ≥ 3 stable pictures were detected. We validated (in a semi-automated way) the position of the paws for each sequence according to the recorded video. At the end of the analysis, we determined the mean weight applied by each paw over time. We used two methods for the extraction of results: (1) the default method, in which all analysed sequences were taken into account, and (2) a method employing a custom filter specific for validated sequences, in which at least one front paw was detected by the sensor.

Supplementary Figures

Supplementary Figure 1: Pedigrees of the nine families that carry the three identified missense variants. The genotypes of the variants and severity of endometriosis (stage I/II or

1 stage III/IV) are provided for each of the 29 genotyped cases, showing missense carrier status for
2 5/5 genotyped cases (G/T) in one family for novel variant C7_34698148 (A), for 11/12
3 genotyped cases (G/A) in four families for rs116825950 (B), and for 6/12 genotyped cases (G/T)
4 in four families for rs34705969 (C).

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Supplementary Figure 2: Regional association plots of common variants in *NPSR1* ± 500Kb and *NPS* ± 500Kb with endometriosis. The probability of an association of disease trait with mutation at a given genetic locus is plotted as $-\log_{10}(\text{P-value})$, y-axis) over genomic location (HG 19, x-axis). The most highly associated biallelic SNP is coloured purple and the other SNPs are coloured according to the strength of LD with the top biallelic SNP by r^2 in the European 1000 Genomes dataset. (A) Association results for *NPSR1* on chromosome 7p14.2 region for overall endometriosis (3194 cases vs. 7060 controls). (B) Association results for *NPSR1* on chromosome 7p14.2 region for familial stage III/IV endometriosis (606 cases vs. 7060 controls). (C) Association results for *NPSR1* on 7p14.2 region for stage I/II endometriosis (1364 cases vs. 7060 controls). (D) Association results for *NPS* on 10p26.2 region for overall endometriosis (3194 cases vs. 7060 controls). (E) Association results for *NPS* on 10p26.2 region for stage III/IV endometriosis (606 cases vs. 7060 controls). (F) Association results for *NPS* on 10p26.2 region for stage I/II endometriosis (1364 cases vs. 7060 controls).

Supplementary Figure 3: *NPSR1* rs142885915 is an expression quantitative trait locus (eQTL) in tissues analysed in the Genotype-Tissue Expression (GTEx) project. Significant association is shown between rs142885915 (Chr7:34855387) and reduced *NPSR1* expression amounts in cerebellum (N=209, Normalised effect size (NES)=-0.58, Ref Allele: GA, Alt allele: G, $p=6.1 \times 10^{-5}$) and suggested association with increased amounts is observed in the cortex (N=175, NES=0.26, Ref Allele: GA, Alt allele: G, $p=0.09$). Data obtained from the GTEx Portal on 27/11/2020.

Supplementary Figure 4: Linkage analysis of the region orthologous to human chromosome 10q26, chromosome 9 in the Rhesus macaque, using SimWalk2. The analysis was conducted in an extended pedigree of 849 rhesus macaques containing 135 affected animals (minimum $P=0.5$).

Supplementary Figure 5: Isotype control staining on eutopic and ectopic endometrial sample sections. The antibodies used against NPSR1 and NPS are of the same isotype, hence the same control was used for both stainings. Sections of eutopic and ectopic endometrium from patients and controls (eutopic) were tested.

(magnification 200x)

Supplementary Figure 6: NPSR1 and its ligand NPS are expressed in the endometrium. (A)

Tissue samples of secretory phase endometrium from patients with minimal/mild (stages I/II) or moderate/severe (stages III/IV) endometriosis were stained with anti-NPSR1 and anti-NPS antibodies, respectively (DAB, brown colour). Endometrium from disease-free controls was stained along with the patient samples. The lining of endometriotic glands (glandular epithelium) stained positive for NPSR1 irrespective of disease status and location of the endometrium (eutopic/ectopic), while the interstitium stained positive for NPS. (Magnification 200x, representative images shown.)

Supplementary Figure 7: NPS and NPSR1 are not expressed in a perivascular pattern.

Serial sections of endometrium were stained with antibodies against the antigens indicated, clockwise from top left: CD31, α -smooth muscle actin, NPS and NPSR1. While NPS and NPSR1 are ubiquitous in the endometrium, with NPSR1 stronger within the glandular epithelium, and no apparent correlation of both NPSR1 and NPS with blood vessels. (Magnification 200x)

Supplementary Figure 8: Gating strategy for evaluation of peritoneal fluid cells. Peritoneal fluid cells were characterised according to FSC/SSC characteristics, single cells were selected and dead cells gated out. NPSR1 was measured against CD45 after adjusting the gates according to isotype controls.

Supplementary Figure 9: The menstrual cycle phase does not influence the percentage of NPSR1⁺ cells in peritoneal fluid immune cell populations in endometriosis patients. Peritoneal fluid cells from endometriosis patients at different phases of the menstrual cycle were measured by CyTOF. The percentages of NPSR1⁺ cells within monocytes, T cells, NK cells and overall CD45⁺ immune cells did not significantly vary with cycle phase. (Follicular phase, n=9; luteal phase, n=11; menstruation, n=6.)

Supplementary Figure 10: Fura Red assay for intracellular calcium (Ca^{2+}) release. Primary human monocytes or THP-1 cells were incubated with Fura Red dye at 37°C for 30 minutes. The cells were seeded into 96-well plates, and Fura Red fluorescence was measured every 0.2 seconds. After a baseline of 2 seconds, the cells were stimulated with NPS at 2.0 seconds, and, as positive control, with ionomycin at 7.0 seconds. The area under the peak evoked by NPS stimulation was quantified against the baseline fluorescence (AUC). N=2-14 wells per concentration; data are mean $\pm$ SEM.

A

B

C

D

E

F

Supplementary Figure 11: The inhibition of NPSR1 reduces peritoneal inflammation *in vivo* in C57BL/6 mice. The efficacy of NPSR1 inhibition *in vivo* was tested in the zymosan induced peritonitis model (ZIP). (A) The experimental schedule had C57BL/6 mice injected

subcutaneously (*s.c.*) with SHA 68R or vehicle before zymosan was injected intraperitoneally (*i.p.*) 1 hour (h) later to induce inflammation. Four hours later, blood samples were taken, mice were sacrificed, and the peritoneal exudate was harvested. Cells were analysed by flow cytometry, cytokines in the plasma were measured by ELISA. (B) Analysis of peritoneal exudate cells (PEC) by flow cytometry with markers for resident macrophages (F4/80) vs infiltrating monocytes (7/4). While injection of PBS did not cause a significant presence of 7/4⁺ cells at 4 h, zymosan injection lead to a shift in populations from resident to infiltrating cells. The degree of inflammation was significantly reduced by SHA 68R (40mg/kg). Plots shown are representative (n= 9 – 12 mice per group). (C), (D) Quantitative analysis of the resident and infiltrating cell numbers showing a dose dependent decrease in inflammatory cell infiltration after injection of SHA 68R (n=10 per group, doses in mg/kg; ** p<0.01, ANOVA with test for dose-dependent trend). (E) The ratio of weight borne on the front vs rear paws was measured 4 h after zymosan challenge and SHA 68R treatment with 40 mg/kg. (F) The analysis of inflammatory cytokines in plasma shows a significant decrease in inflammatory cytokines (IL-4, IL-12p40, IL-17) after inhibition of NPSR1 signalling by SHA 68R (n=8–10 mice per group, doses in mg/kg; data normalised to vehicle-only treatment, log2 up-/downregulation; * p<0.05, ** p<0.01, mixed-effects model analysis with Holm-Sidak correction for multiple comparisons).

A

B

C

D

E

Supplementary Figure 12: The inhibition of NPSR1 reduces inflammatory cytokines and pain in a mouse model of endometriosis. (A) Mice (n=8 per group) were injected with SHA

1 68R at the doses indicated and received 12 pieces of syngeneic uteri into the peritoneal cavity by
2 *i.p.* injection. The pain-induced weight-shifting (DWB) was measured at day 1 and at day 2, and
3 the mice were sacrificed at day 2 to harvest blood and PEC. (B) Representative plots of the cell
4 populations measured within the PEC. (C) Cell numbers of different inflammatory cell types
5 were determined at day 2. ** $p < 0.01$, unpaired t-test. (D) Cytokines measured in the plasma of
6 the mice show a decrease in inflammatory cytokines after treatment with the NPSR1 inhibitor,
7 SHA 68R (data normalised to vehicle-only treatment, * $p < 0.05$, mixed-effects model analysis
8 with Holm-Sidak correction for multiple comparisons). (E) The ratio of weight borne on the
9 front vs rear paws was measured at 24 h (left) and 48 h (right) after zymosan challenge and SHA
10 68R treatment (40 mg/kg).

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Supplementary Tables

Table S1: Results of the aggregate analysis of 7 polymorphic coding variants in *NPSR1* in 3,155 human cases and 2,332 controls.

SNP ID (MAF)	Total cases (N alleles)	Total familial cases (N alleles)	Total familial and stage III/IV cases (N alleles)	Total controls (N alleles)
c7_34698148 (MAF = 0.00009)	6261 G : 1 T	2157 G : 1 T	1215 G : 1 T	4644 G : 0 T
rs116733850 (MAF = 0.00009)	6256 C : 0 T	2158 C : 0 T	1216 C : 0 T	4631 C : 1 T
rs116825950 (MAF = 0.03)	6070 G : 192 A	2081 G : 81 A	1176 G : 44 A	4500 G : 134 A
rs200993440 (MAF = 0.0002)	6261 G : 1 A	2157 G : 1 A	1216 G : 0 A	4647 G : 0 A
rs28480169 (MAF = 0.08)	5767 G : 500 A	1988 G : 170 A	1128 G : 88 A	4310 G : 333 A
rs35436513 (MAF = 0.00009)	6266 G : 0 A	2158 G : 0 A	1216 G : 0 A	4643 G : 1 A
rs77260034 (MAF = 0.0002)	6262 G : 2 A	2156 G : 2 A	1214 G : 2 A	4646 G : 0 A
Aggregate minor allele counts / Total N alleles for 7 SNPs in 4 case sets vs. controls				
1. Cases (n=3155)	696/44170	Chi ² p-value = 0.12		
2. Familial cases (N=1085)	255/15190	Chi ² p-value = 0.04 (Fisher's p=0.04)		
3. Stage III/IV cases (N=1352)	283/18928	Chi ² p-value = 0.61 (Fisher's p=0.59)		
4. Familial and stage III/IV cases (N=613)	135/8582	Chi ² p-value = 0.38 (Fisher's p=0.36)		
Controls (n=2332)	469/32648			

Minor allele counts were calculated as following: Total Alleles for 7 SNPs = 7 SNPs x 2 alleles x N cases and Total Alleles for 7 SNPs = 7 SNPs x 2 alleles x N controls.

Table S2: The 54 polymorphic markers used in fine-mapping of the 7p13-15 linkage region in humans

Marker	Genomic position	Marker	Genomic position*
D7S531	3222261	D7S691	42029530
D7S517	4497914	D7S2428	43249757
D7S513	11651236	D7S667	44502329
D7S507	17597066	D7S2427	44964693
D7S493	21805089	D7S519	46115994
D7S2458	22091028	D7S670	47168970
D7S629	22813900	D7S2506	47680322
D7S673	23848398	D7S674	49539233
D7S2493	24893906	D7S2552	54748618
D7S1791	25142642	D7S2550	55129178
D7S2449	25932780	D7S499	55607412
D7S516	28197694	D7S473	57375730
D7S2515	28711619	D7S494	57481510
D7S2496	29566466	D7S502	67057536
D7S526	30948486	D7S669	77872435
D7S683	34097266	D7S630	88443592
D7S497	34764610	D7S657	92806185
D7S484	35284905	D7S515	101703774
D7S2251	36288502	D7S486	115894674
D7S2209	37559868	D7S530	129202419
D7S528	38028167	D7S640	132639660
D7S2846	38134734	D7S684	138543685
D7S2497	38261492	D7S661	143601894
D7S2507	38900123	D7S636	150699236
D7S510	39189711	D7S798	152811779
D7S2541	40716230	D7S2465	156114684
D7S2548	41744044	D7S2423	157597787

* Human Genome Build: Hg19/NCBI38

Table S3: The 14 markers used for rhesus macaque linkage analyses.

Marker	Genomic position*
Region orthologous to human chromosome 7	
MML3S40	31,520,829
D7S497	34,731,195
MML3S43	35,146,056
MML3S42	35,808,822
MML3S44	44,490,755
D7S2427	45,063,655
D7S2428	57,813,169
Region orthologous to human chromosome 10	
D10S1679	122,798,081
D10S216	125,580,260
D10S1723	125,650,839
D10S575	127,506,309
MML9S30	129,240,996
MML9S29	129,752,837
MML9S32	131,576,710

* Genomic build used: HG19

Table S4: Targeted sequencing results of 10 SNPs in NPSR1 gene in 62 rhesus macaque cases and 49 controls.

rheMac8	Only seen in cases			More prevalent in cases			More prevalent in controls			
Chrom	3	3	3	3	3	3	3	3	3	3
Position	7363529	7366553	7368684	7363528	7366554	7370170	7366446	7366444	7366444	7368677
Ref allele	G	C	G	G	C	A	G	G	G	C
Alt allele	A	T	A	A	T	G	A	A	A	T
Consequence	missense	missense	missense	missense	missense	missense	missense	missense	missense	missense
Plink allele	G	T	A	A	T	G	A	A	A	T
Case frequency	0.045	0.029	0.010	0.315	0.029	0.152	0.000	0.404	0.387	0.236
Control frequency	0.000	0.000	0.000	0.220	0.010	0.146	0.010	0.438	0.444	0.330
Case										
r78102	1/1	0/0	0/0	./.	0/0	0/0	0/0	0/0	0/0	0/0
r78113	1/1	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
r79009	1/1	0/0	0/0	0/0	0/0	0/0	0/0	1/1	./.	0/0
r79066	1/1	0/0	0/0	./.	0/0	0/0	0/0	0/0	0/0	0/1
r79088	1/1	0/0	0/0	1/1	0/0	0/0	0/0	0/0	0/0	0/1
r79123	./.	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/1
r79142	1/1	0/0	0/0	0/0	0/0	0/1	0/0	0/1	0/1	0/0
r79152	./.	0/0	./.	./.	0/0	0/0	0/0	0/0	0/1	0/0
r79165	1/1	0/0	0/0	0/0	0/0	0/1	0/0	0/1	0/1	0/0
r79176	1/1	0/0	0/0	0/1	0/0	0/0	0/0	0/1	0/1	0/1
r80019	1/1	0/1	./.	./.	0/0	0/0	0/0	1/1	1/1	0/0
r80052	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/1
r80100	./.	./.	./.	./.	./.	./.	./.	./.	./.	./.
r80165	./.	./.	0/0	./.	./.	0/0	0/0	./.	./.	0/0
r80176	./.	0/0	0/0	./.	0/0	0/0	0/0	1/1	1/1	0/0
r81011	1/1	0/0	0/0	0/0	0/0	0/0	0/0	1/1	1/1	0/0
r81026	./.	./.	./.	./.	./.	./.	./.	./.	./.	./.
r81036	1/1	0/0	0/0	0/0	0/0	0/1	0/0	1/1	1/1	0/1
r82037	1/1	./.	./.	0/0	./.	./.	./.	./.	1/1	./.
r84042	./.	0/0	0/0	./.	0/0	0/0	0/0	0/0	0/0	./.
r84096	./.	0/0	0/0	./.	0/0	0/0	0/0	0/0	0/0	0/1
r84109	1/1	0/0	0/0	./.	0/0	0/0	0/0	0/1	0/1	0/1
r85133	./.	0/0	0/0	0/0	0/1	0/0	0/0	1/1	1/1	0/1
r88099	0/1	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/1
r90138	./.	0/0	0/0	./.	0/0	0/0	0/0	0/1	0/1	0/0
r90139	./.	0/0	0/0	./.	0/0	0/1	0/0	0/0	0/0	0/1

rh+117	./.	./.	./.	./.	./.	0/0	./.	./.	./.	./.
rh0738	./.	0/0	0/0	./.	0/0	0/0	0/0	0/0	0/0	0/0
rh1424	./.	0/0	0/0	./.	0/0	0/0	0/0	1/1	./.	0/0
rh1676	./.	0/0	0/0	./.	0/0	0/1	0/0	1/1	1/1	0/0
rh1752	1/1	0/0	0/0	1/1	0/0	1/1	0/0	0/1	0/1	0/0
rh1801	1/1	./.	0/0	1/1	./.	0/1	0/0	0/0	0/0	0/0
rh1820	./.	0/0	0/0	./.	0/0	1/1	0/0	0/0	0/0	0/0
rh1831	./.	./.	./.	./.	./.	./.	./.	./.	./.	./.
rh1832	1/1	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
rh1861	./.	0/0	0/0	./.	0/0	0/0	0/0	0/0	0/0	0/0
rh1864	1/1	0/0	0/0	0/0	0/0	0/1	0/0	0/1	0/1	0/0
rhaa31	1/1	0/0	0/1	1/1	0/0	0/0	0/0	0/0	0/0	1/1
rhaa34	1/1	0/0	0/0	1/1	0/0	0/0	0/0	0/0	0/0	0/1
rhaa39	./.	0/0	0/0	0/0	0/0	0/0	0/0	0/1	0/1	0/0
rhaa53	1/1	0/0	0/0	./.	0/0	0/1	0/0	0/1	0/1	0/1
rhaa58	./.	0/0	0/0	./.	0/0	0/0	0/0	0/0	0/0	0/0
rhaa88	1/1	0/0	0/0	0/0	0/0	0/1	0/0	0/1	0/1	0/0
rhaa93	./.	0/0	0/0	./.	0/1	0/0	0/0	0/1	0/1	0/1
rhac77	1/1	0/0	0/0	1/1	0/0	0/0	0/0	0/1	0/1	0/1
rhac80	1/1	0/0	0/0	./.	0/0	0/1	0/0	0/1	0/1	0/1
rhae70	./.	./.	./.	./.	./.	./.	./.	./.	./.	./.
rh-h13	1/1	0/0	0/0	1/1	0/0	0/0	0/0	0/0	0/0	0/0
rh-n86	1/1	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
rh-s18	./.	./.	0/0	./.	./.	0/0	./.	./.	0/0	0/0
rh-t64	./.	0/1	0/0	./.	0/1	0/0	0/0	1/1	1/1	0/0
rh-t92	1/1	0/0	0/0	./.	0/0	0/1	0/0	0/1	0/1	0/0
rh-u16	1/1	0/1	0/0	1/1	0/0	0/1	0/0	0/1	0/1	0/0
rh-u88	1/1	0/0	0/0	./.	0/0	0/0	0/0	0/1	0/1	0/1
rh-v79	./.	0/0	0/0	./.	0/0	0/0	0/0	1/1	1/1	1/1
rh-w20	1/1	0/0	./.	0/0	0/0	0/1	0/0	0/1	0/1	./.
rh-w62	1/1	0/0	0/0	./.	0/0	0/0	0/0	0/1	0/1	0/1
rh-x33	./.	0/0	0/0	./.	0/0	0/0	0/0	0/1	0/1	0/1
rh-y08	./.	0/0	0/0	./.	0/0	0/0	0/0	1/1	1/1	0/1
rh-y30	1/1	0/0	0/0	0/0	0/0	0/0	0/0	0/1	0/1	0/1
rh-y46	./.	./.	./.	./.	./.	./.	./.	./.	./.	./.
rh-y98	./.	0/0	0/0	./.	0/0	0/0	./.	./.	0/0	0/0
Control										
r78007	1/1	0/0	0/0	./.	0/0	0/0	0/0	0/1	0/1	0/0
r78021	1/1	0/0	0/0	0/0	0/0	0/0	0/0	0/1	0/1	0/0
r78024	1/1	0/0	0/0	0/0	0/0	0/0	0/0	0/1	0/1	0/0
r78041	1/1	0/0	0/0	0/0	0/0	0/0	0/0	0/1	0/1	0/0
r78135	1/1	0/0	0/0	./.	0/0	0/0	0/0	0/0	0/0	1/1
r79027	1/1	0/0	0/0	./.	0/0	0/0	0/0	0/1	0/1	0/1

r79093	1/1	0/0	0/0	0/1	0/0	0/0	0/0	1/1	1/1	0/0
r79158	1/1	0/0	0/0	1/1	0/0	0/0	0/0	0/1	0/1	0/0
r80023	1/1	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
r80110	./.	0/0	0/0	0/0	0/0	0/0	0/0	0/0	./.	0/0
r81035	./.	0/0	0/0	./.	0/0	0/1	0/0	1/1	1/1	0/1
r81059	1/1	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
r81130	1/1	0/0	0/0	0/0	0/0	0/1	0/0	1/1	1/1	0/0
r82062	./.	0/0	./.	./.	0/0	1/1	0/0	0/0	0/0	./.
r83029	1/1	0/0	0/0	0/0	0/0	0/0	0/0	0/1	./.	0/1
r84049	./.	0/0	./.	./.	0/0	0/0	0/0	1/1	1/1	./.
r84089	./.	0/0	0/0	0/0	0/1	0/0	0/0	1/1	1/1	0/1
r84101	1/1	0/0	0/0	1/1	0/0	0/0	0/0	0/0	0/0	0/1
r85106	1/1	0/0	0/0	1/1	0/0	0/0	0/0	0/0	0/0	1/1
r88092	1/1	0/0	0/0	1/1	0/0	0/0	0/0	0/0	0/0	1/1
r89114	1/1	0/0	0/0	1/1	0/0	0/0	0/0	0/0	0/0	1/1
r89167	1/1	0/0	0/0	./.	0/0	0/1	0/0	0/0	0/0	0/1
r90143	./.	0/0	0/0	./.	0/0	0/0	./.	./.	./.	1/1
r90146	./.	./.	0/0	./.	./.	./.	0/0	0/1	0/1	0/0
r90147	1/1	0/0	0/0	./.	0/0	0/0	0/0	0/1	0/1	0/1
r91048	./.	0/0	0/0	./.	0/0	0/0	0/0	0/0	0/0	0/1
r92076	1/1	0/0	0/0	0/0	0/0	0/1	0/0	0/1	0/1	0/0
r92098	1/1	0/0	0/0	./.	0/0	0/0	0/0	0/1	0/1	0/1
r92103	1/1	0/0	0/0	0/0	0/0	0/0	0/0	1/1	1/1	0/0
r93047	1/1	0/0	0/0	0/0	0/0	0/1	0/0	1/1	1/1	0/1
rh1436	1/1	0/0	0/0	./.	0/0	0/1	0/0	0/0	0/0	0/1
rh1505	1/1	0/0	0/0	./.	0/0	0/0	0/0	0/0	0/0	0/0
rh1657	1/1	0/0	0/0	0/0	0/0	0/0	0/0	1/1	1/1	0/0
rh1808	1/1	0/0	0/0	0/0	0/0	0/1	0/0	1/1	1/1	0/0
rh1821	1/1	0/0	0/0	0/0	0/0	0/0	0/0	0/1	0/1	0/0
rhaa62	1/1	0/0	0/0	./.	0/0	0/1	0/0	0/1	0/1	0/1
rhaa83	./.	0/0	0/0	./.	0/0	0/0	0/0	0/0	0/0	1/1
rhac88	1/1	0/0	0/0	0/0	0/0	0/1	0/0	0/1	0/1	0/1
rhad86	1/1	0/0	0/0	./.	0/0	0/1	0/0	0/1	0/1	0/1
rhaj10	1/1	0/0	0/0	./.	0/0	0/0	0/0	0/1	0/1	0/1
rhao37	1/1	0/0	0/0	0/0	0/0	0/0	0/1	0/1	./.	0/0
rhao41	1/1	0/0	0/0	./.	0/0	0/0	0/0	0/1	0/1	0/0
rh-q72	1/1	0/0	0/0	0/0	0/0	0/1	0/0	1/1	1/1	0/0
rh-s49	1/1	0/0	0/0	./.	0/0	0/0	0/0	0/1	0/1	0/0
rh-s63	1/1	0/0	0/0	0/0	0/0	0/0	0/0	0/1	0/1	0/0
rh-t39	1/1	0/0	0/0	./.	0/0	0/0	0/0	0/0	0/0	0/1
rh-y11	1/1	0/0	0/0	./.	0/0	0/0	0/0	0/1	0/1	0/0
rh-z18	1/1	0/0	0/0	./.	0/0	0/0	0/0	0/1	0/1	1/1
rh-z69	1/1	0/0	0/0	./.	0/0	0/1	0/0	0/0	0/0	0/1

Table S5: Markers used for the separation of peritoneal cell populations by viSNE algorithm (63).

Marker	Conjugate	Marker	Conjugate
CD3	Er170Di	CD56	Yb176Di
CD4	Nd145Di	CD64	Nd146Di
CD8	Dy162Di	CD68	Yb171Di
CD9	Yb172Di	CD94	Yb174Di
CD11b	Lu175Di	CD117	Nd143Di
CD11c	Tb159Di	CD127	Sm149Di
CD14	Gd160Di	CD163	Ho165Di
CD16	Nd148Di	CD206	Er168Di
CD20	Sm147Di	FceR1a	Eu153Di
CD40	Nd150Di	NPSR1	Dy164Di
CD45	Sm154Di	VEGF	Nd142Di

1 **Table S6: Peritoneal Fluid samples analysed by CyTOF.**

Patient No.	Disease Stage	Menstrual Phase	Patient No.	Disease Stage	Menstrual Phase
19	Control	Follicular	49	Stage I	Irregular
20	Control	Menstruation	50	Stage I	unknown
21	Control	Follicular	51	Stage I	Menstruation
22	Control	Pill	52	Stage I	No Periods
23	Control	Irregular/no cycle	53	Stage II	Luteal
24	Control	Amenorrhea/unknown	54	Stage II	Follicular
25	Control	unknown	55	Stage II	Menstrual
26	Control	Pill/no cycle	56	Stage II	Menstruation
27	Control	Luteal	57	Stage II	Follicular
28	Control	No periods	58	Stage II	Pill
29	Control	Luteal	59	Stage II	Irregular
30	Control	Luteal	60	Stage II	No periods
31	Control	Follicular Phase	61	Stage II	Follicular phase
32	Control	Follicular Phase	62	Stage II	unknown
33	Control	Irregular	63	Stage III	Follicular/Luteal
34	Control	Luteal Phase	64	Stage III	Menstruation
35	Control	Luteal phase	65	Stage III	Irregular
36	Control	unknown	66	Stage III	Luteal Phase
37	Stage I	Unknown	67	Stage III	No Periods
38	Stage I	Follicular	68	Stage IV	Luteal
39	Stage I	Unknown	69	Stage IV	Pill/Luteal
40	Stage I	Menstruation/Luteal	70	Stage IV	unknown
41	Stage I	Follicular	71	Stage IV	Luteal
42	Stage I	Menstruation	72	Stage IV	Luteal
43	Stage I	Luteal/ovulation	73	Stage IV	Luteal/Follicular
44	Stage I	Follicular	74	Stage IV	Follicular Phase
45	Stage I	Luteal/pill effect	75	Stage IV	Irregular
46	Stage I	Follicular	76	Stage IV	Luteal Phase
47	Stage I	Menstruation	77	Stage IV	Follicular phase
48	Stage I	Irregular			

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	Control	Endometriosis	Stage I	Stage II	Stage III	Stage IV
Follicular	4	9	4	3	1	2
Luteal	5	11	3	1	1	6
Menstruation	1	6	3	2	1	0
Other/unknown	8	14	6	4	2	2

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