Genetic variability in exon 1 of the glucocorticoid receptor gene *NR3C1* is associated with postoperative complications

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Abstract. Patients undergoing major surgery experience postoperative inflammation, which may contribute to postoperative morbidity. Endogenous glucocorticoids (GCs) are an essential part of the stress response, but this response varies between individuals, which may in turn affect clinical outcome and specifically postoperative inflammation. Exon 1 of the *NR3C1* gene, encoding the GC receptor (GR), contains an established region of differential regulation. DNA methylation patterns in this region have been found to differ between individuals. The present study investigated the methylation status and genotype in the cytosine-phosphate-guanine (CpG) island in exon 1 of *NR3C1* in 24 patients [Median age 65.5 (range 42-81) years, 11 male, 13 female] who underwent major abdominal (12 pancreatic, 12 hepatic) surgery and explored its association with postoperative complications. DNA was extracted from peripheral blood leukocytes and underwent targeted bisulfite sequencing of the CpG island. Complications were graded according to the Clavien-Dindo classification and 14 out of 24 patients had postoperative complications. Multifactorial and partial least square analyses were used to analyse the data. A homogenous demethylated pattern was observed in all patients and no single CpG methylation was associated with postoperative complications. Four SNPs were significantly associated with higher Clavien-Dindo scores. Genetic variability in the chromosome 5:143,402,505-143,405,805 region of exon 1 of the GR gene *NR3C1*, but not DNA methylation, was associated with more severe postoperative complications in patients having major abdominal surgery. These results indicated that the patients' response to GCs may be of clinical importance for inflammatory conditions.

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Abbreviations: GR, glucocorticoid receptor; GC, glucocorticoid; *NR3C1*, nuclear receptor subfamily 3 group C member 1; SNP, single nucleotide polymorphisms; CpG, cytosine-phosphate-guanine; PLS, partial least squares; chr, chromosome; BiS, bisulfite; TF, transcription factor; GRCh38/hg38, Human Genome Reference Consortium, human (build) 38 (human genome 38)

Key words: glucocorticoid receptor, nuclear receptor subfamily 3 group C member 1, single nucleotide polymorphisms, surgery, postoperative complications, epigenetics, DNA methylation

Introduction

Patients undergoing major surgery, for example abdominal cancer surgery, experience a clinically well-known phenomenon of postoperative inflammation (1). This inflammation, together with haemodynamic and metabolic instability, contribute to postoperative morbidity (2).

Endogenous glucocorticoids (GCs) are an essential part of the stress response in mammals and serve important roles in immunological and haemodynamic homeostasis and metabolism (3). Deficient, or excessive, GC-signalling leads to debilitating disease, especially under physiological stress (4,5). In states of hyperinflammation, such as autoimmune disease, synthetic GCs are used therapeutically. However, although widely used and comprehensively studied, the molecular mechanisms of GCs have not been fully explored (6). The response to GCs is also highly variable between individuals and the cause of this diversity remains unknown (7).

In our previous study it was demonstrated that responsiveness to GCs may be related to a patient's ability to recover from surgically induced inflammatory stress. In patients undergoing major abdominal surgery, a negative correlation between the regulation of the glucocorticoid receptor (GR)α gene nuclear

receptor subfamily 3 group C member 1 (*NR3C1*), in peripheral blood leukocytes, and the length of stay in the intensive care unit was observed (8). The GC signalling pathway is intricate. Several GR isoforms exist and have been investigated in relation to numerous conditions. These investigations have revealed a diverse regulation of downstream target genes (9-11). The gene *NR3C1*, encoding the GR, consists of nine exons and is evolutionarily conserved. Exon 1 comprises nine different variants that are transcribed, but not translated (12). Of these nine non-coding exon 1 variants, seven are situated closely together and contain a cytosine-phosphate-guanine (CpG) island spanning 3 kb (Fig. 1), an established site for differential regulation.

Methylation patterns in exon 1 differ between individuals (13). Differences in methylation in this region have been demonstrated to influence various forms of stress responses in humans and mice (12,14-16). The methylation pattern is manifested early in life and later affects susceptibility to stressors in neuropsychological, as well as other, conditions (17-20). DNA methylation of *NR3C1* has not been explored in settings of postoperative inflammatory stress. A translational approach is warranted, allowing for the investigation of inter-individual differences in the molecular biology of the GR gene in relation to a clinically well-described state of inflammation.

We therefore hypothesise that there is an association between GR gene *NR3C1* exon 1 methylation status and postoperative complications following major abdominal surgery. In the present study, the methylation status and genotype in the CpG island at *NR3C1* exon 1 in the peripheral blood of patients who subsequently underwent major abdominal surgery was explored. Correlations with postoperative complications were further investigated.

Materials and methods

Patients. Ethical approval for the present study was granted by the Stockholm Regional Ethical Review Board (Stockholm, Sweden; approval no. 2009/366-31). Patients eligible for inclusion were those >18 years, accepted and planned for surgery involving resections of the pancreas or liver, pre-operatively estimated to last >4 h. Patients requiring long-term GC treatment pre-operatively or with any known disease of the glucocortid system were excluded.The patients recruited for the present study were enrolled after informed consent was obtained, orally and in writing, in accordance with The Declaration of Helsinki. Whole blood (4 ml) was collected from 24 patients who were to have major abdominal (pancreatic or liver) surgery. Samples were drawn during a pre-operative visit at the Department of Surgery, Karolinska University Hospital Huddinge (Stockholm, Sweden) in 2014. The present study did not allow for a power calculation of the required number of participants, as the distribution of SNPs in the patient population was not known.

Outcomes. Complications were graded according to Clavien-Dindo classification (21), which is a well-established grading system in the surgical community. It is an ordinal scale, ranging from 1 to 5, with 1 denoting only a minor deviance, from the expected postoperative course, i.e., additional antiemetic treatment. Grade 2 denotes need for pharmacological therapy not considered routine or standard post-operative treatment. Grade 4 denotes organ failure in any organ system and grade 5 denotes mortality of the patient due to any cause. Grade 3 signifies complications, such as anastomic leakage or wound dehiscence, necessitating re-intervention and is divided into two sub-classes depending on whether this re-intervention is performed without (3a) or with (3b) general anaesthesia. Complications graded ≥3b are regarded as major.

Sampling and DNA extraction. Pre-operative samples of whole blood were collected in EDTA tubes (BD Vacutainer; BD Diagnostics) and the erythrocytes were immediately treated with cold (4-6˚C) ammonium lysis buffer (153 mM NH_4Cl, 10 mM $KHCO_3$ and 0.01 mM EDTA adjusted to pH 7.4-7.5) until tranluscent. After lysis and washing in PBS, leukocytes were resuspended in 350 μl of RLT Buffer from the Qiagen RNEasy kit (Qiagen AB) and were subsequently frozen. DNA was extracted using the Qiagen RNEasy kit in a QIAcube (Qiagen AB) according to the manufacturer's protocol. Concentration and purity assessments were performed using a NanoDrop 1000 (Thermo Fisher Scientific, Inc.). The 260 nm/280 nm absorbance ratios were in the range of 1.75-1.85.

Targeted bisulfite (BiS) sequencing. The CpG island is located at chromosome (chr)5:143402505-143405805 [Human Dec 2013 (GRCh38/hg38) Assembly], i.e., GRCh38/hg38, Human Genome Reference Consortium, human genome build 38 (human genome 38) (12). Targeted BiS sequencing was performed by Zymo Research Corp. Samples containing approximately 400-1400 μg DNA were diluted and BiS-converted using the EZ DNA Methylation-Lightning kit (Zymo Research Corp.) at 98˚C for 8 min and 54˚C for 60 min. BiS-converted DNA was then PCR-amplified using ZymoTaq PreMix (Zymo Research Corp.) for library construction, using a microfluidics system (Fluidigm Access Array; Fludigm Corporation), the specific cycling conditions and final concentrations are proprietary to Zymo Research Corp. The primer sequences used are presented in Table SI. BiS-converted, amplified DNA underwent next generation sequencing using the Illumina MiSeq platform (Illumina, Inc.) and corresponding kits by Zymo Research Corp. A paired-end 300 bp configuration was used and libraries were loaded at 8 pM concentrations. The raw data was aligned for CpG methylation calling. Sequence reads were identified using standard Illumina base-calling software. Sequence alignment was made if the coverage exceeded 10 reads. Furthermore, in addition to the CpG methylation analysis, the full sequences were analysed for SNPs to the extent allowed for following BiS conversion using Bioconductor software package R (version 3.7, https://bioconductor.org/news/bioc_3_7_release/) with the packages Rqc (version 1.10.2), Rsamtools (version 1.35.2), msa version 1.12.0 and GenomicRanges (1.32.0).

Multifactorial analysis. Multifactorial analysis was done in R (version 3.5.0) (22). Consensus sequences from each patient were extracted from the sequence data obtained via BiS-sequencing, which used a threshold of 0.5 as minimum probability threshold in the multiple sequence alignment package (23). All biological parameters were assessed using

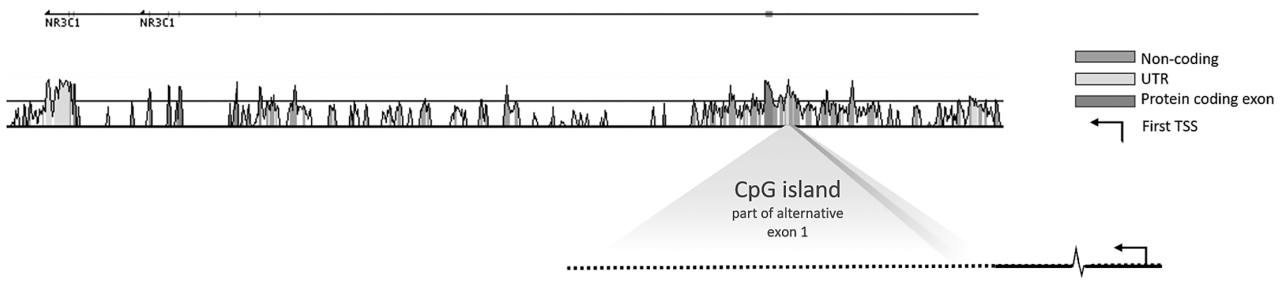

Figure 1. *NR3C1* gene structure. Representative image (VISTA plot) of the glucocorticoid receptor gene *NR3C1*. The histogram demonstrates conservation between the human and mouse genes and the topmost line denotes protein coding exons. Conservation is defined as nucleotide sequences >100 bp with >70% conservation. Magnification demonstrates the CpG island containing 319 CpG sites. *NR3C1*, nuclear receptor subfamily 3 group C member 1; CpG, cytosine-phosphate-guanine; TSS, transcription start site; UTR, untranslated region.

multiple factor analysis in Factominer (version 2.4) (24). This multifactorial approach was selected as it enabled the analysis of individual and combined SNP effects, methylation patterns, patient characteristics and clinical data. No patient outliers skewed the model. No individual factors, except for the Clavien-Dindo classification, were able to explain the significant portion of the variance observed. Therefore, all other factors were excluded from further analysis.

Parameters with minimal risk of non-biological bias were used to construct the statistical model, including liver or pancreas surgery, age, sex, and CRP levels. Factors omitted at this stage were those including some degree of subjective assessment, for example ASA-classification or pre-operative chemotherapy. Those variables were instead subsequently fitted into the model as supplementary variables. Patients with postoperative complications (defined as Clavien-Dindo ≥1; n=14) were included for partial least squares (PLS) analysis which was performed using the PLS package. PLS was chosen because it can accept ordinal variables, including Clavien-Dindo classification. Moreover, Clavien-Dindo classifies postoperative complications and therefore the non-differentiation between patients without complications presented a lower boundary of measurement. This truncation of the outcome variable, complications (graded according to the Clavien-Dindo classification), produced a region with zero variance that disqualified it from appropriate factor analysis. Therefore, it was only possible to analyse the severity of complications and patients without complications were excluded from further analysis.

The Clavien-Dindo classification system was used as the response variable and all investigated nucleotide positions with more than zero variance were used as the predictor matrix. 'Leave one out' cross-validation in Factominer (version 2.4) was used to avoid overfitting and the number of components with the smallest mean square error of prediction was retained (24). Two components explaining 71% of the variance were retained. Scores were used as an estimate of contribution of each SNP. P<0.001 was considered to indicate a statistically significant difference and was used as the cut-off value.

Statistical analysis. Data are presented as medians with ranges or interquartile ranges, as appropriate. Analysis of differential allele comparisons was performed using an independent-samples Mann-Whitney U Test (SPSS version 26; IBM Corp.). P<0.05 was considered to indicate a statistically significant difference.

Bioinformatics. Genomic positions determined to be significant were acquired from the statistical analysis and were investigated using the University of California, Santa Cruz (UCSC) Genome Browser Human Dec 2013 (GRCh38/hg38) Assembly (12). A VISTA plot was used to visualise the gene (25). Allele frequencies (MAFs) were examined in the 1000 Genomes Phase 3, ESP and gnomAD (v2.1) cohorts (26-28)

Results

Patients. Patient characteristics and clinical data are presented in Table I. The median age distribution was 65.5 (range 42-81) years and sex distribution was 11/13 male/female. Overall, 14/24 patients had a postoperative complication. The specimen histopathology demonstrated different clinical conditions of the liver or pancreas, as listed in Table I.

Of the parameters available for factor analysis, sex and benign disease were statistically significant (P<0.05) in dimension one and two with 7.1 and 6.7% of the variance, respectively. These effects were not, however, assessed to skew the model and consequently not considered in subsequent analyses. Hence, a multifactorial PLS regression-based approach was employed to assess the association of methylation with postoperative complications, using the full BiS-converted sequencing data and the Clavien-Dindo classification system only.

DNA methylation and postoperative complications. When examining all 319 CpG islands in the BiS-converted sequence, a homogeneous demethylated pattern was demonstrated. The whole region was demethylated, except for the last CpG island (position, chr5:143405794). There was no association between CpG methylation at this site and postoperative complications (Fig. 2).

SNPs associated with postoperative complications. The combined effect of multiple contributing nucleotide sites was assessed, taking the clinically relevant heterogeneity into account. The results demonstrated that 4 positions were significantly associated with postoperative complications (Fig. 2). These four sites were previously reported to be SNPs. The properties of these SNPs are presented in Table II, along with the

Table I. Patient characteristics.

No.	Age	Sex[a]	H (cm)	W (kg)	WBC billion (cells/l)	Op. type	ASA[b]	Op. dur.[b] (min)	Chemo[b]	CD[b]	HDU (days)[b]	ICU[b]	CRP D1 (mg/l)	CRP Max D1-3 (mg/l)	Histo.[a]
1	60	F	177	91.6	6.1	Panc	1	197	No		5	No	46	146	NET
2	65	F	164	56.6	8.4	Panc	2	383	No	3b	5	No	39	39	IPMN
3	76	F	172	67.1	5.8	Panc	3	352	No	4	3	No	55	127	NET
4	70	F	167	78.6	5.0	Panc	2	423	No	2	6	No	45	139	PDAC
5	59	M	176	104.4	5.2	Panc	3	290	No		4	No	48	192	NET
6	76	M	175	79.5	7.4	Panc	2	197	No	2	6	No	29	92	IPMN
7	78	M	180	70.0	6.0	Panc	2	315	No	3	6	No	50	90	IPMN
8	45	F	168	82.0	6.7	Panc	1	176	No		2	No	28	183	IPMN
9	70	F	176	68.2	6.9	Panc	2	314	No		5	No	54	98	PDAC
10	66	M	176	79.5	8.7	Panc	2	366	No		3	No	49	102	PDAC
11	81	F	169	67.0	5.6	Panc	3	283	No		2	No	57	82	PDAC
12	63	F	170	101.7	8.3	Panc	3	205	No	4,5	2	Yes	35	66	PDAC
13	72	M	182	94.6	4.7	Hep	3	250	No	3b	2	No	25	177	CRCM
14	65	M	176	96.2	7.5	Hep	2	414	No	2	8	Yes	17	80	HCC
15	70	M	169	74.8	6.7	Hep	3	290	Yes	2	4	No	19	80	CRCM
16	62	F	173	76.7	7.8	Hep	2	105	No		2	No	17	111	Benign
17	57	M	191	88.8	10.2	Hep	2	280	Yes	3b	5	No	84	129	CRCM
18	59	M	173	72.9	7.5	Hep	2	269	No	4a	2	Yes	30	179	GIST
19	68	M	176	89.9	3.9	Hep	2	213	Yes		2	No	N/A	47	CRCM
20	47	F	170	81.0	7.4	Hep	2	288	No	1	0	No	32	48	Benign
21	70	F	165	62.0	7.4	Hep	3	210	No		2	No	23	88	HCC
22	58	F	173	74.6	4.8	Hep	2	275	No	3,5	2	No	19	134	Benign
23	69	M	187	92.0	9.5	Hep	2	227	No		2	No	67	146	Chr. infl.
24	42	F	160	49.0	5.3	Hep	1	343	Yes	5	2	Yes	33	96	CRCM

Twenty-four patients were included in the present study. In the factor analysis variables sex and benign disease were statistically significant ([a]$P<0.05$) in dimension one and two with 7.1 and 6.7% of the variance, respectively. [b]Variable considered as 'organisational' and therefore analysed as a supplementary variable with no influence on the factor analysis performed. F, female; M, male; H, height; W, weight; WBC, pre-operative white blood cells; op. type, type of operative procedure; panc, pancreatic; hep, hepatic; ASA, American Society of Anesthesiologists physical system class; op. dur., duration of operation; chemo, pre-operative neo-adjuvant chemotherapy; CD, Clavien-Dindo grade (blank=no complications); HDU, high-dependency unit-length of stay; ICU, admission to Intensive Care Unit; CRP D1, C-reactive protein level on day 1; CRP Max D1-3, highest C-reactive protein level on days 1-3; Histo, histopathological diagnosis; NET, neuroendocrine tumor; IPMN, intraductal papillary mucinous neoplasia; PDAC, pancreatic ductal adenocarcinoma; CRCM, colorectal adenocarcinoma metastasis; HCC, hepatocellular carcinoma; GIST, gastrointestinal stromal tumour; chr. infl., chronic inflammation. N/A, none available.

Table II. Summary of genomic loci significant in PLS.

ID	Position (chr5)	% cons.	NCBI dbSNP accession no.	Allele freq. in ref. seq.	m.a. effect	m.a. CDs (≥1)	TF binding sites
SNP1	143402837 (353[a])	0	rs10482614	G/A (-) strand (88,5/11,5 (C 0,04))	CpG del	2 of 14 (2/24)	SMARCA4, TFAP2, STAT1
SNP2	143404507 (2023[a])	99	rs1039242888	G/C unknown[b]	CpG del	1 of 14 (1/24)	SMARCA4, TFAP2C, STAT1, RBL2, EGR1
SNP3	143404514 (2030[a])	95	rs904759782	G/C unknown[b]	CpG del	6 of 14 (10/24)	SMARCA4, TFAP2C, STAT1, RBL2
SNP4	143404564 (2080[a])	99	rs3806855	A/C (88,5/11,5)	Unknown	7 of 14 (8/24)	SMARCA4, TFAP2C, STAT1, RBL2

[a]In parentheses, relative position in the sequenced fragment sequenced. [b]SNP information without frequency data Position, University of California, Santa Cruz Genome Browser on Human Dec. 2013 (GRCh38/hg38) Assembly; % cons, percentage conservation from '30 mammals conservation by PhastCons (27 primates; phastCons30way)' University of California, Santa Cruz Genome browser; NCBI dbSNP accession no., NCBI (National Center for Biotechnology Information) dbSNP (data base for Single Nucleotide Polymophisms) accession number; PLS, partial least squares analysis; chr, chromosome; freq., frequency; seq., sequence; ref., reference; m.a., minor allele; CDs, Clavien-Dindo score; (CDs column denotes how many of patients with a Clavien-Dindo graded complication had minor allele carriership. The fraction in brackets show minor allele carriership amongst all patients in the study.) TF, transcription factor; TFAP2C, transcription factor AP-2γ; RBL2, RB transcriptional corepressor like 2; SMARCA4, SWI/SNF related matrix-associated actin-dependent regulator of chromatin subfamily a member 4; EGR1, Early growth response protein 1; GRCh38/hg38, Human Genome Reference Consortium, human (build) 38 (human genome 38).

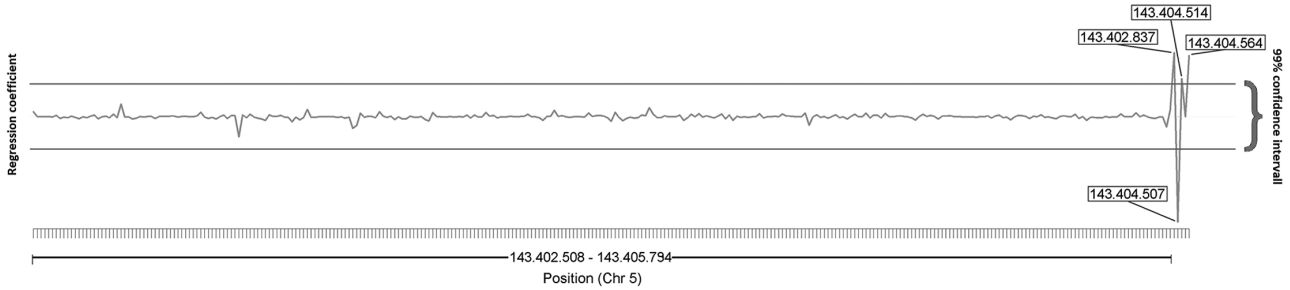

Figure 2. Impact on Complications (Clavien-Dindo classification) of each methylation site and SNPs. Partial least square analysis. The impact of each methylation site and SNP on clinical outcome (as defined by the Clavien-Dindo classification). Numbering on the x-axis displays the position of each methylation site analysed. The boxed number markings are SNP sites and represent numbers relative to the sequencing start. Overall, 71% of the variance was explained using this model. Lines represent the 99% confidence interval. Significant positions are marked by the boxed numbers. Chr, chromosome.

summarised data from these aforementioned studies [obtained using the UCSC genome browser (Fig. S1)]. For ease, the SNPs are numbered 1-4. Of the four SNPs identified in our analysis, two were previously identified common SNPs; NCBI (National Center for Biotechnology Information) dbSNP (data base for Single Nucleotide Polymophisms) accession number rs10482614 (chr5:143402837; SNP1) and NCBI dbSNP accession number rs3806855 (chr5:143404564; SNP4). GRCh38/hg38 demonstrated that the highest population minor allele frequencies (MAFs; observed in any population, including the 1000 Genomes Phase 3, ESP and gnomAD cohorts (26-28) were 0.26 and 0.25, respectively. SNP1 consists of a minor allele (A), which deletes a possible methylation site (CpG) (16). SNP4 has previously been described to affect promotor activity (16). The same nucleotide as in SNP4 can also be part of a three-nucleotide deletion, (NCBI dbSNP accession number rs796817133), highest population MAF <0.01), which was not encountered in our cohort (29). NCBI dbSNP accession number rs1039242888 (chr5:143404507; SNP2) has not been thoroughly investigated but has previously been reported to have been encountered (highest population MAF 0.02). This SNP, SNP2, was only present in one patient in the present study and should therefore be interpreted with caution. NCBI dbSNP accession number rs904759782 (chr 5:143404514; SNP3) was previously reported to have a highest population MAF <0.01 but was not further investigated and therefore its clinical significance is unknown. SNP3 was fairly common in the present study with over 40% of the patients being carriers of this minor allele. The minor alleles of SNPs 1-3 all result in depletions of CpG sites. Bioinformatics data on transcription factor (TF) binding sites at these four specific positions were obtained, revealing five different TFs: EGR1, SMARCA4, TFAP2C, STAT1, and RBL2. These are listed in Table III along with their mechanisms.

Major vs. minor SNP alleles. The PLS analysis was repeated containing each allele (major or minor) for each SNP, one at a time and the findings from the pooled PLS analysis, as

Table III. Summary of TFs.

TF	Definition	Function
EGR1	Early growth response protein 1	Transcriptional regulator of genes involved in differentiation and mitogenesis
RBL2	RB transcriptional corepressor like 2	Tumor suppressor gene
SMARCA4	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4	Regulation of chromatin structure
STAT1	Signal transducer and activator of transcription 1	Transcription activator (upon cytokine and growth factor signaling)
TFAP2C	TF AP-2γ	Sequence specific DNA-binding TF implicated in gene regulation development and differentiation

TF, transcription factor.

presented above were confirmed. Both homozygotes and heterozygotes for the minor alleles were included in the minor allele group. The influence of major vs. minor allele carriership on postoperative complications was compared and of the four SNPs which emerged significant in our PLS (above), only SNP4 was significantly different between the major and minor alleles (p=0.016) when analysing one factor, i.e. major vs minor allele carriership for this one SNP, at a time (Fig. 3).

Discussion

In the present study, it was demonstrated that GR gene *NR3C1* exon 1 genotype was associated with postoperative complications (using the Clavien-Dindo classification) in patients undergoing major abdominal surgery. However, the results also demonstrated that DNA methylation in the CpG island of the *NR3C1* exon 1 had no impact on clinical outcome. Moreover, this region proved to be highly demethylated in all patients included in the study. This unexpected finding contradicted the hypothesis and contrasts with the high variance in methylation of this region found in a population of healthy individuals (13). The observed enrichment in demethylation of the CpG island in the *NR3C1* gene in the studied cohort merits further investigation, for example, by comparing healthy volunteers.

The sequenced region (chr5:143,402,505-143,405,805) contained 13 previously described SNPs (with a frequency of >1% in the population). Of these common SNPs, four were noted as being associated with postoperative complications in the present study. SNP1 (NCBI dbSNP accession number rs10482614), located in-between exon 1H and 1C, has previously been investigated (16). SNP1 can eliminate a CpG site; however, it was not found to be correlated with the methylation of the 1H promotor, which is connected to haemodynamic stress responses (17). Furthermore, SNP1 is associated with psychiatric disorders and affects the relative expression levels of different mRNA isoforms (25). In the present study, SNP1 was also correlated with SNP4 (NCBI dbSNP accession number rs3806855), in an allelic associative manner, as previously reported (16). There is therefore evidence that these SNPs have a biological function and could possibly harbour clinically relevant functions. Furthermore, in addition to SNP1 and SNP4, two more SNPs emerged in the present study. SNP3 was significantly associated with postoperative complications. This SNP has previously been recorded but not studied (rs1039242888). In the present study SNP2 was identified in a

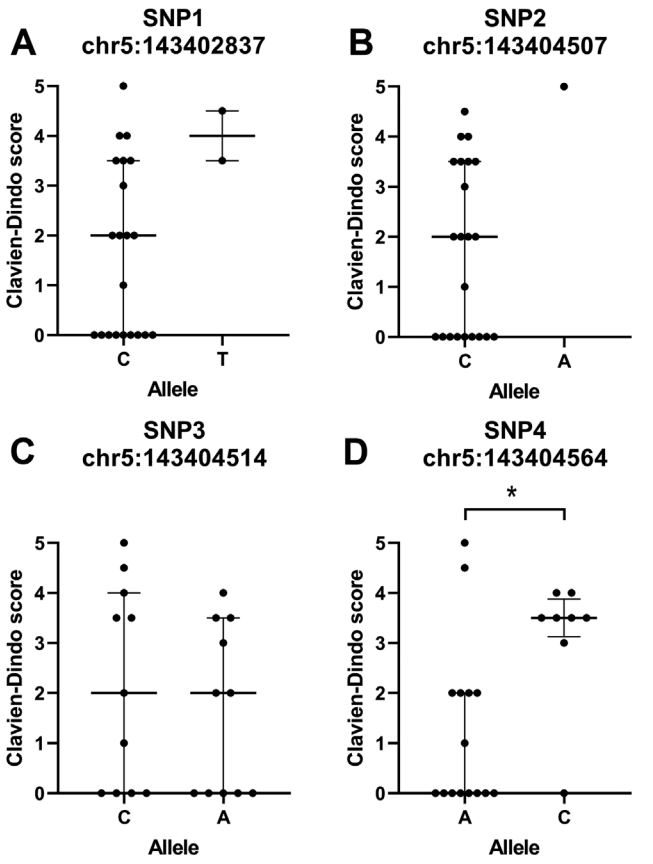

Figure 3. Major vs. minor allele carriership and postoperative complications. Postoperative complications, classified according to the Clavien-Dindo system, in carriers of the major vs. minor alleles of SNP1-4. Patients with no Clavien-Dindo score are labelled as zero. SNP1,2 and 4, n=24; SNP3, n=23. (A) chr5:143402837, SNP1; (B) chr:143404507, SNP2; (C) chr5:143404514, SNP3; and (D) chr5:143404564, SNP4. Data are presented as the mean ± inter-quartile range. SNP4 is significantly different between the major and minor alleles (P=0.016). Major alleles are shown on the left, minor alleles in the right. Chr, chromosome; A, adenine; C, cytosine; T, Thymine; *P<0.05.

single patient with a major complication (biliary anastomosis leakage).

SNP2-4 are closely situated within 60 bp from one another, making this region particularly interesting when investigating the regulation of the *NR3C1* gene. The frequencies of SNP3 and SNP4 were remarkably high in the small patient cohort of the present study, >40% and 33%, respectively, compared with the previously reported highest population MAF of carriers of the minor allele (GRCh38/hg38). Similar to the marked enrichment of demethylation of the CpG site, the high frequency of SNP3 and 4 in our patient cohort merits further investigation.

The high degree of conservation in the region of SNP2-4, along with the multiple binding sites of TFs, has proven this to be an important area of regulation of GR expression. Other studies have reported SNPs and demonstrated their different GR transactivation potentials (30-33). Associations between GR polymorphisms and disease activity in chronic inflammation have previously been reported (34). Both risk and severity of acute graft vs. host disease following transplantation of haematopoietic stem cells, are shown to be affected by the presence of certain SNPs in either recipients or donors (35). GR SNP results from numerous studies have been compiled in a review concluding that there is substantial evidence of GR SNP effects on clinical phenotypes (36).

The altered frequency of SNPs reported in the present study may have impacted the binding affinity and avidity of TFs and thus affected subsequent GR mRNA transcription. It has previously been demonstrated that methylation in this region can counteract transcriptional effects of certain common SNPs, possibly compensating for genotype alterations (17). Furthermore, there was a trend towards an association with postoperative complications for carriers of the SNP1 minor allele (P=0.087), which in the present study was correlated with SNP4. This finding is similar to previous reports (16,32). Furthermore, for SNP2, which is located close to SNP4, there was a similar trend for patients with the minor allele (P=0.083).

Overall, the results suggested that multiple levels of GR expression regulation may be associated with the severity of postoperative complications, including the genetic variants in exon 1, and that this regulatory region within the *NR3C1* gene may be of relevance in a clinical setting. Furthermore, genetic variability of the *NR3C1* gene is not infrequent in the general population and it is therefore unlikely that it has a noticeable impact on healthy individuals (www.ncbi.nlm.nih.gov/SNP) (37). However, in the present study an association has been demonstrated with an adverse clinical outcome after surgically induced inflammatory stress.

A limitation of the present study was the lack of comparisons to healthy volunteers. There were also shortcomings in statistical power, but these findings can be useful for power calculations in future studies. However, the present study was an exploratory study and to the best of our knowledge is the first of its kind, combining gene sequencing, clincal data on surgical outcomes and advanced statistics. Furthermore, similar translational studies are needed to elucidate patterns of GR regulation in patients with normal as well as complicated outcomes in the context of inflammatory responses.

In conclusion, the association between genetic variability in GR gene *NR3C1* exon 1 and postoperative complications in patients undergoing major abdominal surgery indicates that the GC response may be of importance for inflammatory responses that affect clinical outcomes. The observed enrichment of demethylation of the CpG island in exon 1 of the *NR3C1* gene in patients planned for major surgery warrants further clinical studies.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request. According to Swedish law, clinical individual data can be shared only if Ethical Approval is obtained as human subjects are involved. The sequencing data generated in the present study may be found in the European Genome-phenome Archive (EGA), under accession number EGAS00001005737 (ega-archive.org).

Authors' contributions

TG, LS, ACW and OW conceived the study. TG, LS, MW, EAB and LKS were responsible for data curation. MW, EAB and TG confirm the authenticity of all raw data, MW and TG performed data analysis. OW, ACW, LS and EAB acquired funding for the project. TG, EAB and MW performed the investigation. TG, MW, EAB, OW, ACW and LKS designed the methodology. LS, ACW and OW provided resources. LS, ACW and OW were responsible for project administration. MW was responsible for the software used in the project. LS, ACW, OW and LKS supervised the project. EAB and MW validated the results. MW and EAB visualized the study. EAB and TG prepared the original manuscript draft. TG, MW, ACW and LS wrote, reviewed and edited the manuscript. All authors read and agreed to the final manuscript.

Ethics approval and consent to participate

Ethical approval for this study was granted by the Stockholm Regional Ethical Review Board (Stockholm, Sweden; approval no. 2009/366-31). The patients recruited for the present study were enrolled after informed consent was obtained, orally and in writing, in accordance with the Declaration of Helsinki.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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