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Increased oxidative stress with substantial dysregulation of genes related to oxidative stress and DNA repair after laparoscopic colon cancer surgery

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ABSTRACT

Surgical stress is followed by oxidative stress, where reactive oxygene species may act as regulators of pathways related to cancer cell survival and metastatic ability. Furthermore, reactive oxygene species may cause DNA and RNA damage.

The aim of this study was to examine whether laparoscopic colon cancer surgery causes oxidative stress and dysregulation of related pathways.

Methods: Patients undergoing elective laparoscopic surgery for colon cancer were included. Blood and urine samples were drawn on the day prior to surgery and on day 1 and 10 after surgery.

Results: Twenty-six patients were included.

Out of 140 genes previously identified as sensitive to regulation by reactive oxygene species, 46 were significantly differentially expressed on day 1 after surgery (FDR < 0.05). Upregulated genes were related to cellular immune suppression, proliferation, migration and epithelial to mesenchymal transition. Downregulated genes were related to IFN pathways and cytotoxic immunological reactions.

Genes related to DNA repair were primarily downregulated on day one after surgery, and urinary excretion of 8oxdG was decreased on day two after ($p = 0.004$), and increased on day 10 after surgery ($p = 0.01$).

Conclusion: Laparoscopic colon cancer surgery causes oxidative stress, and impaired DNA repair. Gene expression profiling indicates that reactive oxygen species may act as regulators of pathways related to increased risk of metastasis and cellular immune suppression after surgery. Measures of intracellular oxidative stress, indicates impaired DNA repair on day two after surgery, and sustained oxidative stress on day 10 after surgery.

1. Introduction

Colorectal cancer (CRC) is the third most common malignancy in the world, accounting for over 600,000 deaths annually [1]. Surgery is essential for cancer treatment, and surgical removal of the tumor is mainstay of treatment in a curative treatment strategy [2]. Even after minimally invasive surgery for CRC performed on curative intent, there is a continued risk of cancer relapse of up to 25–30% within the first five years after surgery [3].

Growing evidence supports that surgery and surgical stress after cancer surgery contribute to an increased risk of cancer relapse [4]. Surgery causes shedding of tumor cells [5], and micro metastasis might be present, even after curative surgery [6]. These residual cancer cells gain enhanced growth and metastatic abilities in the postoperative period due to surgical stress [7]. Many factors in the perioperative period may facilitate this risk of recurrence after surgery including activation of the sympathetic nervous system [8,9], impaired cellular immune function [10], and inflammation [11].

High levels of reactive oxygen species (ROS) have been proposed to be a key component in the unwanted postoperative cascade of oncogenic potential following cancer surgery [12].

ROS are products of normal mitochondrial respiration, and other

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basic cellular functions, and play a key role in many signaling pathways such as MAP kinase and NF-κB [13]. Oxidative stress classically results from an imbalance between the antioxidant defense mechanisms and excess production of ROS in a state of inflammation or stress, and a complex and compartmentalized organizational structure is now evident [14]. Moderate oxidative activation provides numerous regulatory changes whereas massive oxidative stress leads to redox signaling and ROS induced damage that has been associated with many disease processes, including cancer development [15,16].

Redox signaling is where ROS acts as regulators of genetic pathways. ROS then induces changes in expression of genes that play a key role in many steps necessary for cancer cell metastasis including epithelial to mesenchymal transition (EMT) [17], cancer cell invasion [18], adhesion [19], and angiogenesis [20]. Several animal and *in vitro* studies indicate an association between postoperative ROS and increased risk of residual cancer [21–23].

The aim of this study was to investigate whether surgical stress, caused by laparoscopic colon cancer surgery causes oxidative stress and impacts redox signaling and DNA-repair mechanisms.

2. Methods

2.1. Participants

From January to July 2016, patients undergoing elective, curative intended laparoscopic surgery for colon cancer stage I-III Union for International Cancer Control (UICC) performed in an Enhanced Recovery After Surgery (ERAS) [24] setting at Zealand University Hospital in Denmark, were consecutively included in the study. Patients undergoing neoadjuvant therapy, or patients with immune defects, previous cancer, or patients who experienced postoperative infectious complications, were excluded.

2.2. Settings

Eligible patients received information regarding the study and were included after giving oral and written consent. Patients followed standard of care for colon cancer in an ERAS setting both prior to, and after surgery. No restrictions were imposed on pain management, anesthesia or surgical approach.

2.3. Data collection and processing

Demographic data was collected through questionnaires and electronic patient charts including age, gender, smoking status, medical history, body mass index (BMI), American Society of Anesthesiologist (ASA) scores and Charlson Comorbidity Index-score (based on three categories according to comorbidities: CCI 0: none, 1: one, 2: two 3: ≥ three) and UICC stage (based on preoperative CT scans and histology results).

All postoperative complications up to the day of last samples were registered within the Clavien Dindo Classification system [25].

2.4. Blood samples

The genetic pathways that can be regulated by ROS – redox sensitive genes, and genes related to human DNA repair mechanisms are well described [26,27]. Gene expression profiling of whole-blood provides a possibility to gain insight to changes in transcription of multiple genes in all immune cells, and whole-blood gene expression profiling (WBGP) has previously been described as a method for exploring oxidative stress and changes in gene expression after surgery [28].

Blood samples for WBGP were collected on the day prior to surgery, and POD 1 and 10. The specific days were based on a previous study published by this group, showing most significant changes in gene expression on day 1 after surgery, and expression similar to preoperative expression on POD 10 [29]. Blood samples were collected in Paxgene tubes (Preanalytix, Hombrechtikon, Switzerland) stored at −80 °C until analysis. After sampling, tubes were stored at room temperature for 24 h, then at −20 °C for one day, and finally transferred to −80 °C. Total RNA was extracted using the Paxgene Blood RNA kit (Qiagen, Franklin Lakes, NJ, USA). The quantity of RNA was tested with a NanoDrop spectrophotometer ND-8000 (NanoDrop Technologies), and RNA quality was tested with an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA).

The GeneChip™ WT PLUS Reagent Kit was used to convert 500 ng purified total RNA to biotin-labeled cDNA which was fragmented and hybridized to Affymetrix GeneChip™ Human Transcriptome Array 2.0.

2.5. Urine samples

A well-described modification caused by ROS interaction with DNA and RNA. In DNA, the modification is a C-8 hydroxylation of deoxyguanine, and the related DNA repair mechanism involving p53 and single nucleotide replacement, is measurable in urine, where oxidized guanine is excreted as 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG). In RNA, the modification is also a C-8 hydroxylation of guanine, but there are no repair mechanisms. The damaged RNA is recognized upon translation, cut from the RNA string, and excreted in the urine, where can be measured as part of the excreted string 8-oxo-7,8-dihydroguanosine (8-oxoGuo) [30]. These products are validated as biomarkers of intracellular oxidative stress. The analysis offers a downstream analysis of oxidative stress, and in relation to DNA also provides information on whether there is a functioning repair mechanism [31].

Urine samples were collected pre-operatively and POD 1, 2, and 10 in sterile urine sample kits with no additives. Samples were kept on ice and centrifuged at 4 °C at 1.590*g* for 15 min. Hereafter, 1.5 mL was transferred to Eppendorf-tubes and kept at −80 °C until analysis. Analysis was performed using ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS), the method and validation has been described in detail elsewhere [32]. The values of 8-oxodG and 8-oxoGuo were reported as 8-oxodG/creatinine and 8-oxoGuo/creatinine to correct for varying voided urine volumes.

2.6. Statistics

2.6.1. Gene expression profiling

Background correction, normalization, and gene expression index calculation of probe intensities were done in Affymetrix Expression Console software using the robust multi-array average (rma) method. To calculate the significance of difference in gene expression between pre and postoperative samples, the regularized limma *t*-test for paired data was applied. P-values were corrected for multiple hypothesis testing using the false discovery rate (FDR), and an FDR < 0.05 was considered as statistically significant. Genes included in the analysis were chosen, based on current literature [26,27]. (Appendix 1 includes a full list of genes). The whole gene set was corrected for multiple hypothesis in all selections of genes (33.803).

The pairwise fold changes of gene expression were calculated for postoperative samples compared to preoperative samples. The gene expression fold change matrix was visualized by heat maps using the heatmap.2 function embedded in the *gplots* R-package.

2.6.2. Excretion of 8oxodG and 8-oxoGuo

A linear mixed effect model was used to determine statistical differences in pre and postoperative 8-oxodG and 8-oxoGuo values in urine samples using the *lme* function embedded in the *nlme* R-package. In this model, individually included patients were included as a random effect variable designated “person”, and “day” was included as a fixed effect variable in the model. P-values less than 0.05 was considered statistically significant.

2.7. Ethical considerations

The Central Committee for Health Research and Ethics (file no: 2008-58-0020) and the Danish Data Protection agency (protocol: SJ567) approved the study which was conducted according to the Declaration of Helsinki. Gene expression data has been deposited into Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo; accession no. GSE122626).

3. Results

A total of 34 patients completed the study. Eight patients were excluded, one had benign disease and seven experienced a postoperative complication within the first 10 days after primary surgery, including four patients who had an anastomotic leakage (CD3b), one had ileus (CD2) and two experienced a postoperative wound infection found on day 10 after surgery (CD1 and 2 respectively).

The 26 patients included in the final analyzes all underwent laparoscopic colon cancer surgery in an ERAS regime, followed standard of care with no infectious postoperative complications, and no other postoperative complications above CD1. Patients were omitted from hospital after a mean of 2,4 days (see Table 1 for patient demographics).

3.1. Gene expression profiling

Analysis of the expression of 33.803 genes identified 5756 significantly differentially expressed genes with an FDR <0.05, comparing preoperative expression to postoperative day 1. Comparing preoperative expression to day 10, no genes were significantly differentially expressed.

Identifying 147 genes related to DNA repair, showed that 46 genes were significantly differentially expressed between samples from the preoperative day versus postoperative day 1. Most of these genes were downregulated, 34 vs 12 upregulated (Fig. 1).

Table 1
Demographics for patients undergoing laparoscopic colonic resection for colon cancer.

Age, mean (interval)		66,8 (50–84)
Gender n (%)	Male	16 (61,5)
	Female	10 (38,5)
ASA-score n (%)	1	3 (11,5)
	2	21 (80,8)
	3	2 (7,7)
BMI n (%)	18.5–24.9	9 (34,6)
	25–29.9	8 (30,8)
	>30	9 (34,6)
Smoking n (%)	Current smoker	3 (11,5)
	Former smoker	12 (46,2)
	Never smoker	11 (42,3)
Alcohol (drinks/week) n (%)	0-14/21	21 (80,8)
	>14/21	5 (19,2)
Comorbidity n (%)	0	18 (69,2)
	1	3 (11,5)
	2	2 (7,7)
	Missing	3 (11,5)
Performance status n (%)	0	21 (80,8)
	1	3 (11,5)
	2	2 (7,7)
UICC n (%)	1	9 (34,6)
	2	10 (38,5)
	3	7 (26,9)
Anesthesia n (%)	Intravenous	18 (69,2)
	Inhalation	8 (30,8)
Laparoscopic procedure n (%)	Right hemicolectomy	6 (23,1)
	Transverse colectomy	1 (3,8)
	Left hemicolectomy	1 (3,8)
	Sigmoidectomy	17 (65,4)
	Complete colectomy	1 (3,8)

ASA: American Society of Anesthesiologist Sore, BMI: Body Mass Index.

Analysis of changes in expression of 141 genes sensitive to redox signaling showed 46 significantly differentially expressed genes. Heatmap clustering of fold changes in expression of these genes on postoperative day one and ten compared to preoperative, showed a two-sided clustering with upregulated genes related to inflammation, migration, adhesion, adaptive immune suppression and inflammation in one cluster, and downregulated genes related to antigen presentation and general adaptive immunity in the other cluster (Fig. 2). (Fold changes and short descriptions of functions of the protein products of the individual genes included and depicted in the heatmap analysis, are described in Appendix 2).

3.2. Excretion of 8oxodG and 8-oxoGuo

Before surgery, 8oxodG was 1,6 nMol/mMol creatinine (SD: 0,8), and on the first postoperative day it was 3,0% higher, 1,6 nMol/mMol (SD: 0,8 $p > 0,05$). Day two after surgery, excretion was 18,1, % lower than preoperative (1,3 nMol/mMol; SD: 0,5; $p = 0.03$) and on day 10, excretion was 13,7% higher than before surgery (1,8 nMol/mMol, SD: 0,9; $p = 0.005$) (Fig. 3a).

Mean excretion of 8-oxoGuo before surgery was 2,5 nMol/mMol creatinine (SD: 1,6) and on the first postoperative day it was 2,7 nMol/mMol (SD: 1,3), only 3,0% higher than preoperative ($p > 0,05$). Day two after surgery, excretion was 13,7% lower than preoperative (2,2 nMol/mMol; SD: 0,7 $p > 0,05$), and on day 10 after surgery, excretion was 11,9% higher than preoperative excretion (2,8 nMol/mMol, SD: 1,4; $p > 0.05$), (Fig. 3b).

4. Discussion

In 26 patients undergoing elective, laparoscopic colon cancer surgery with a curative intent in an ERAS setting and without any infectious postoperative complications, we found an increased oxidative stress response and accompanying dysregulation of redox-sensitive genes. Previous animal studies have found a correlation with oxidative stress and increased risk of recurrence after surgery [12]. The findings in this clinical study supports this hypothesis.

4.1. DNA-repair mechanisms after surgery

On the first day after surgery, there was a significant downregulation of most genes related to DNA repair mechanisms (34 out of 46 were downregulated), including tumor suppressor TP53 and gluthatione peroxidase genes [33,34]. The significant downregulation indicated that DNA repair may have been dysfunctional on the first postoperative day after surgery.

This was also indicated in the analysis of urinary excretion of biomarkers of oxidative stress. There was a significantly lower excretion of 8oxodG on day two after surgery, indicating impaired DNA repair mechanisms. There was a higher excretion on day 10 after surgery, indicating a sustained higher oxidative stress compared to pre-operative values. Changes in 8oxoguo (biomarker of oxidative RNA damage) followed the same pattern as 8oxodG, but remained insignificant.

The excretion of 8oxodG has been accepted as a biomarker for oxidative stress [35], and it is well known that the excretion of products of DNA repair, caused by oxidative stress, is dependent on a functional DNA repair mechanism [30]. Therefore, our findings of lower excretion of waste-products from DNA repair, on day two after surgery, correlates with our findings that 34 out of 46 genes related to DNA repair were significantly downregulated on the first postoperative day.

There was a significantly higher excretion of 8oxodG on day 10 after surgery, indicating a higher oxidative stress than before surgery, though at the same time, none of the genes related to oxidative DNA repair or redox sensitive genes were significantly dysregulated.

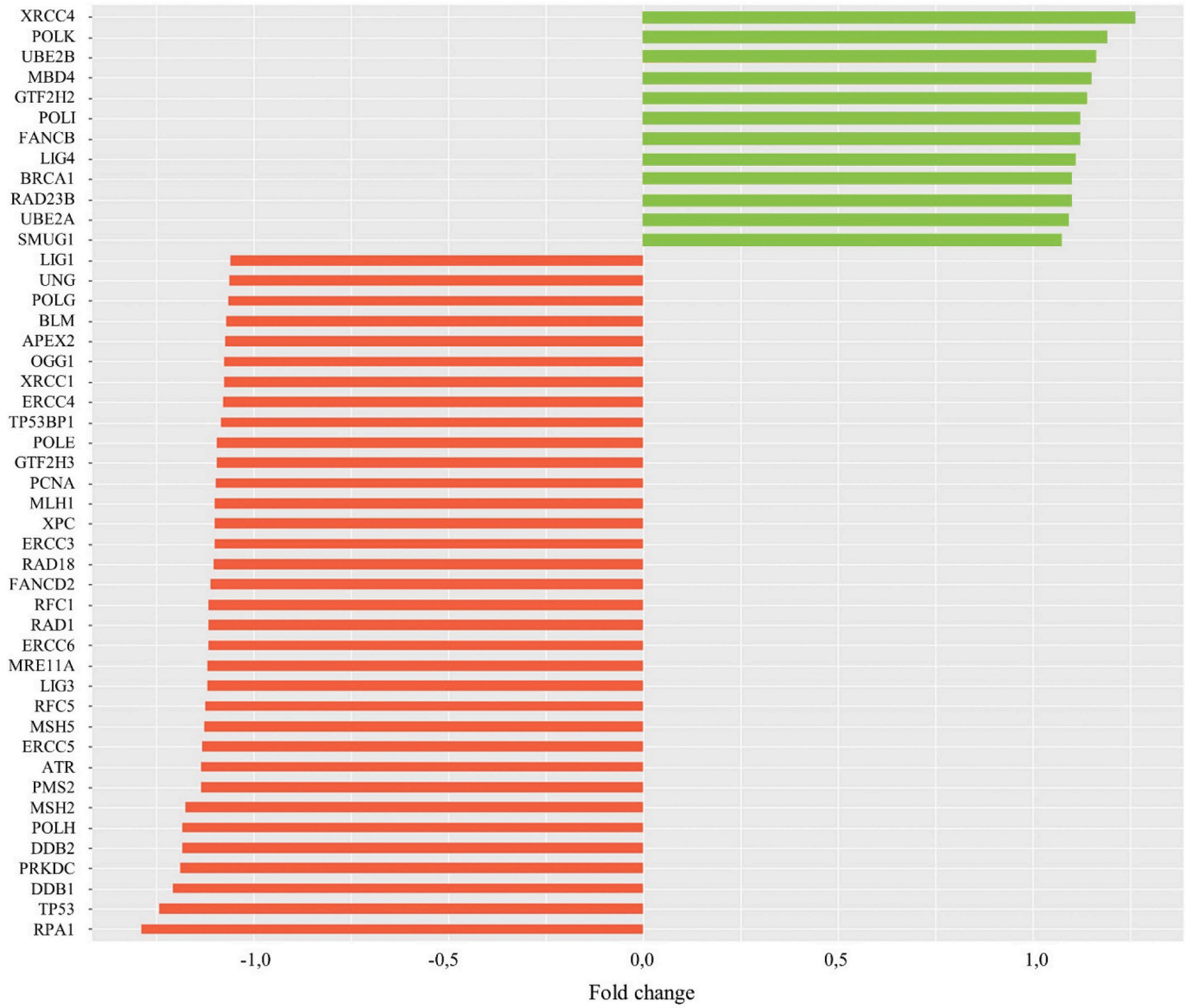

Fig. 1. Fold changes of significantly dysregulated DNA repair genes on first postoperative day, compared to preoperative.

4.2. Expression of redox-sensitive genes after surgery

Heatmap clustering analysis of changes in genes sensitive to redox signaling, indicated that oxidative stress may be a component in the association between surgical stress and increased risk of cancer relapse. Amongst genes clustering together, genes encoding integrins (ITGAM, ITGAX, ITGB2) were upregulated. Integrins promote cell-extracellular matrix adhesion that are essential for leucocyte adhesion and migration, but also increase adhesion and migration in circulating cancer cells [36]. Several Heat shock protein family genes were also upregulated after surgery (HSPA1A, A1B and A6). The protein products of these genes are involved in protein homeostasis and enhance cell survival following cellular stress and furthermore, it is well known that cancer cells rely on this system for survival [37]. Many genes related to proliferation, migration and EMT were upregulated (PTP4A1, TXN, RPS6KA1, JUNB) [38]. Genes involved in the innate immune system and suppression of the adaptive immune system were also amongst the genes that were upregulated after surgery, including IL1B, IL10, CEBPB, Lyn and STAT5B. IL1B is a mediator of innate immunity, cell proliferation, differentiation, and apoptosis. Upregulation is closely associated with EMT and invasiveness of various cancers including CRC [39]. IL10 downregulates the expression of Th1 cytokines, MHC class II antigens through initiation of regulatory T-cells and can block NF-kappa B activity [40]. The protein product of the CEBPB gene is related to initiation of the acute phase response [41], and Lyn has an inhibitory role in myeloid lineage proliferation and has been implicated in a variety of human tumors including CRC [42]. Expression of transcription factor STAT5B was also upregulated after surgery. STAT5B plays an important role in the function and development of Tregs and is associated with a suppression of antitumor immunity and an increase in proliferation [43]. Amongst the redox-sensitive genes that were downregulated after surgery where STAT1, 2, 4 and JAK1 that are all genes involved in IFN related pathways and Th1 cytotoxic immunological reactions. CSF which encodes the protein product colony stimulating factor which is involved in macrophage differentiation and function was also downregulated together with ZAP 70 and Lck which plays a major role in initiation of cellular immunity and TCR signaling. Lastly, the important tumor suppressor gene TP53 encoding p53 that responds to cellular stresses to regulate expression of target genes, thereby inducing cell cycle arrest, apoptosis, senescence, DNA repair, or changes in metabolism was significantly downregulated.

It has previously been proposed, that redox signaling is a key player

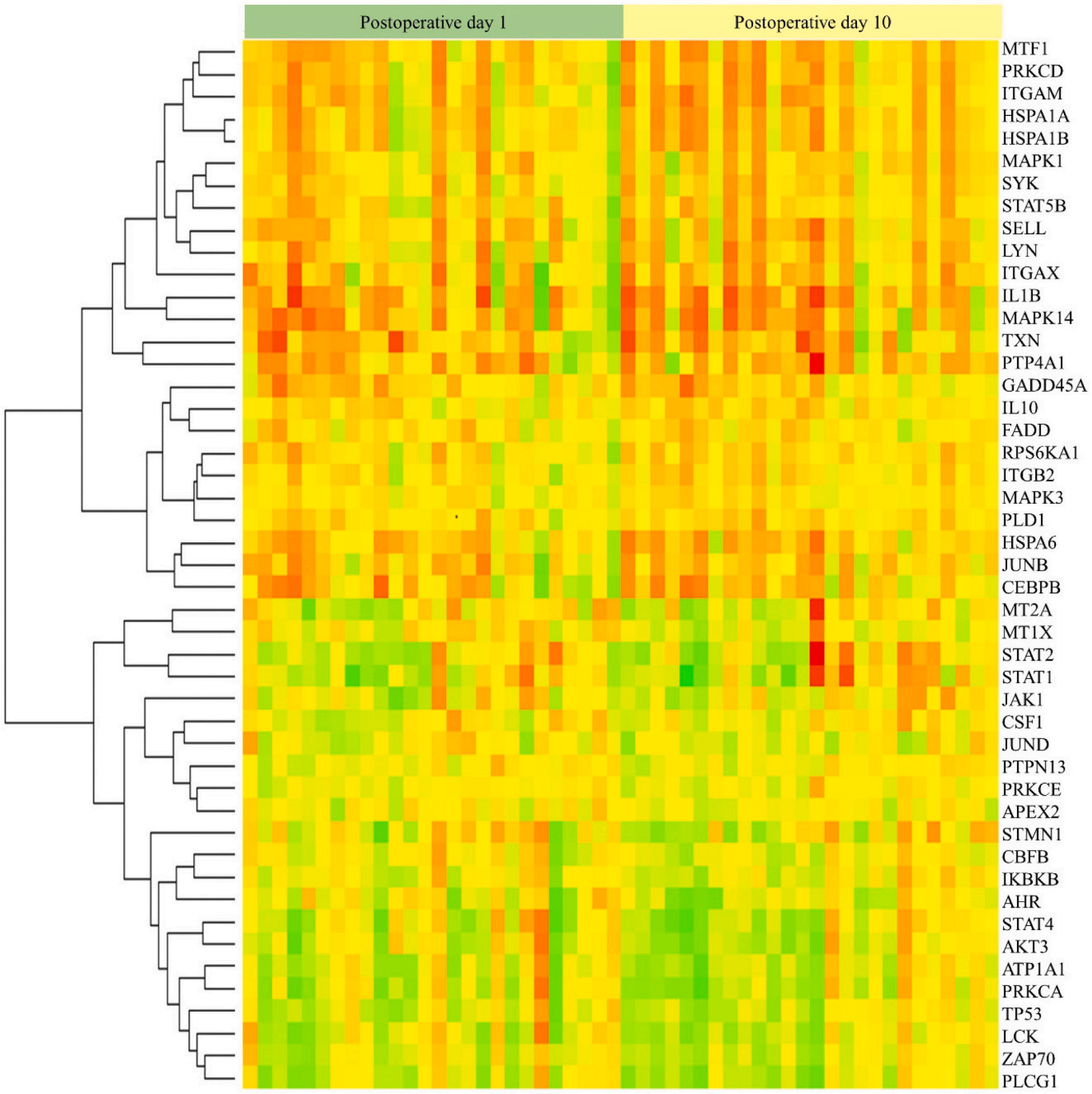

Fig. 2. Heatmap of fold changes in expression of redox sensitive genes on postoperative day one and ten, compared to preoperative gene expression values.

in cancer recurrence following surgical tumor resection [12]. Laparoscopic surgery has been associated with less systemic oxidative stress than open surgery in a systematic review comparing open versus laparoscopic abdominal surgery [44], and in a clinical trial with 60 patients randomized to open versus laparoscopic colectomy [45]. A clinical trial randomizing 19 patients to open versus laparoscopic sigmoid resection did, however, not find a significant difference in oxidative stress [46]. In the present study, we only investigated laparoscopic surgery. The results indicate increased redox signaling and decreased expression of DNA repair defense genes in the immediate postoperative period.

The findings of the present study are supported by experimental studies that find a correlation with oxidative stress after surgery, and increased risk of metastasis. Experimental studies have found that surgery-induced oxidative stress was responsible for increased cancer cell adhesion and formation of intracellular gaps, exposing ECM to adherence molecules for cancer cells in the liver [22], and increasing binding sites for tumor cells on the endothelium [21]. Scavenging of ROS has led to diminished peritoneal tumor recurrence and decreased tumor cell adhesion in animal studies [23].

The perioperative period is of growing interest in regard to surgical stress and possible intervention towards an increased carcinogenic potential in this crucial period [8]. The findings in the present study indicate that oxidative stress might be a key component in regard to carcinogenic effects of cancer surgery, and highlights the importance of future intervention studies in the perioperative period, which could focus on decreasing oxidative stress [47]. The present study is

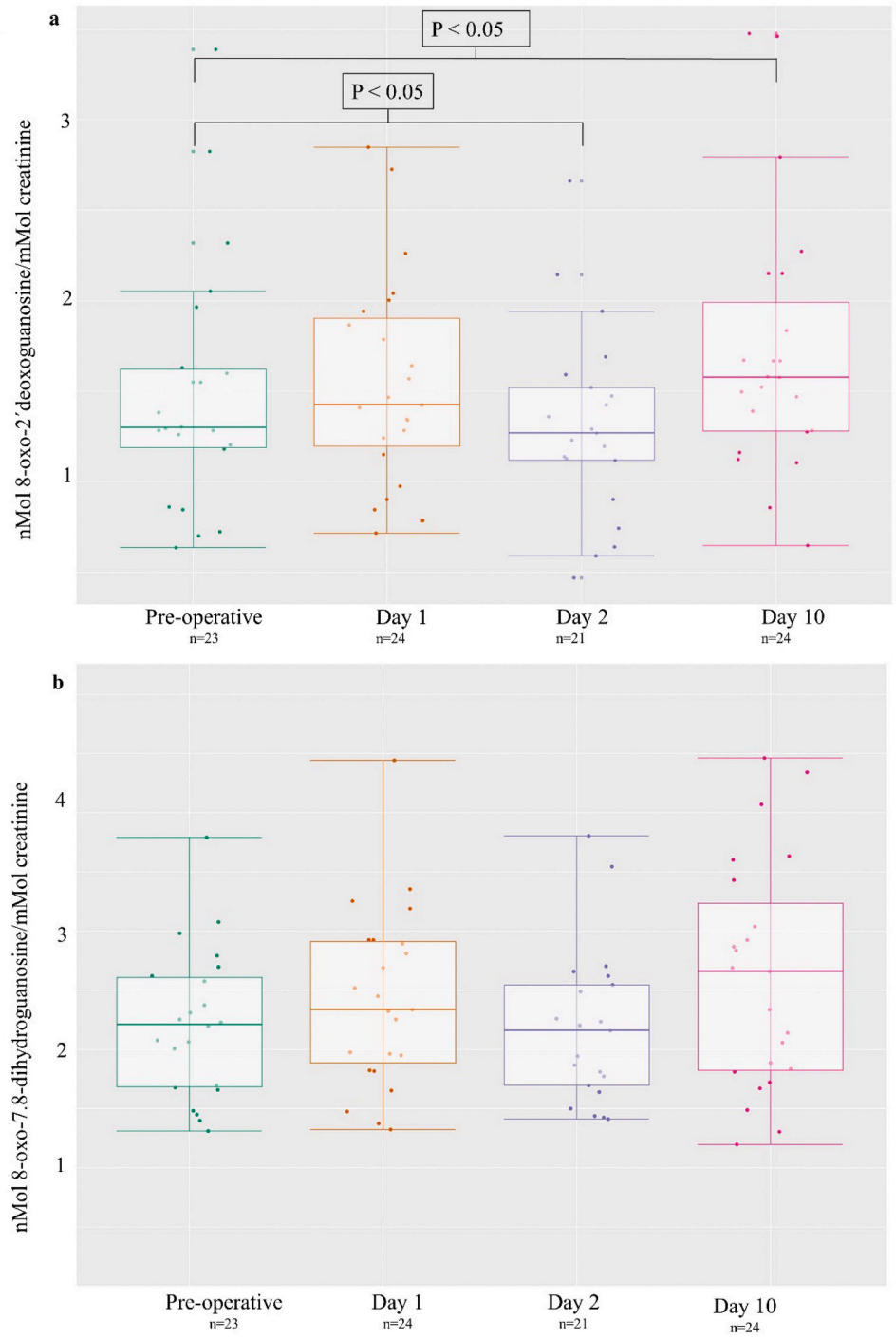

Fig. 3. a and b: Level of 8-oxodG (a) and 8-oxoGuo (b) measured in urine samples before surgery and day 1, 2 and 10 after surgery.

explorative, and does not have power to investigate correlation between recurrence, surgical and oxidative stress. This should be investigated in a larger prospective study.

The results of this study indicates, that laparoscopic colon cancer surgery caused oxidative stress and impaired DNA repair in the immediate postoperative period after curatively intended laparoscopic colon cancer surgery.

Further studies focused on long term consequences and possible interventions and mechanisms are warranted.

Founding

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Ethical Approval for Research

Yes.

External Funding

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Author statement

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Declaration of competing interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.suronc.2020.06.009.

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