

## Plasma Concentration of the Lipid Peroxidation (LP) Biomarker 4-Hydroxynonenal (4-HNE) in Benign and Cancer Patients

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**Abstract.** *Background/Aim:* The present study investigated the plasma concentration of the lipid peroxidation (LP) biomarker 4-hydroxynonenal (4-HNE) in benign and cancer patients having the rectus sheath block (RSB) analgesia after midline laparotomy. Plasma concentrations of catalase (CAT) and malondialdehyde (MDA) were used as a reference. *Patients and Methods:* This study assessed three LP biomarkers; CAT, MDA and 4-HNE and compared the plasma levels to the patient satisfaction 24 h postoperatively (SFS<sub>24</sub>; 0=fully unsatisfied; 10=fully satisfied); the overall pain at rest (NRS<sub>r</sub>) and when pressing the wound at 20 Newton force (NRS<sub>p</sub>) were surveyed and filed on a 11-point numeric rating scale at 24 h following surgery (NRS; 0=no pain; 10=worst pain). There were 56 patients in the study, of whom 12 were excluded due to missing plasma samples. The final study cohort consisted of 15 patients with benign disease and 29 patients with cancer. *Results:* The RSB analgesia enhanced significantly the SFS<sub>24</sub> scores in the study groups ( $p=0.001$ ). The plasma 4-HNE decreased immediately after operation (POP1) and the postoperative decrease between the preoperative and the POP1 values in the 4-HNE marker were statistically significant

( $p<0.001$ ). The individual plasma 4-HNE and MDA concentration correlated significantly in benign and cancer patients ( $r=0.413$ ,  $p<0.001$ ). *Conclusion:* The present study confirms the applicability of the plasma biomarker 4-HNE to cast further light on the postoperative pain in midline laparotomy patients.

Failure in antioxidant defenses could lead to formation of reactive oxidative species (ROS) and lipid peroxidation (LP) (1-9). The uncontrolled ROS is called oxidative stress (OS) and is a common feature in cancer cells (1). OS in cancer cells could be demonstrated by an increase in OS markers and ROS in cancer cells can be counteracted by substances known as antioxidants: vitamin E and C, flavonoids and carotenoids (10, 11). The most important antioxidative enzymes are catalase (CAT) (12, 13), glutathione peroxidase (GPX1) (4, 14) and superoxide dismutase (SOD1) (15, 16). The role of LP in etiology of cancer, neurodegenerative and cardiovascular diseases, has motivated researchers to clarify the mechanism of LP and to develop biomarkers (7, 17-19). LP proceeds by three mechanisms; (i) free radical oxidation, (ii) free radical independent non-enzymatic oxidation, and (iii) enzymatic oxidation (1, 2). Polyunsaturated fatty acids (PUFAs) and cholesterol are oxidized through enzymatic and non-enzymatic routes. Malondialdehyde (MDA) and 4-Hydroxynonenal (4-HNE) are the ROS that cause OS in cells, targeting the subcellular structures to form covalent protein adducts (1, 2). 4-HNE is an  $\alpha,\beta$ -unsaturated hydroxyalkenal that is produced by LP in cells. 4-HNE has 3 reactive groups: an aldehyde, a double-bond at carbon 2, and a hydroxy group at carbon 4 (1, 2).

As far as we know, the differences in the LP stress biomarker 4-HNE plasma concentrations in midline

This article is freely accessible online.

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*Key Words:* Benign disease, cancer, lipid peroxidation, plasma 4-hydroxynonenal, pain score following surgery.

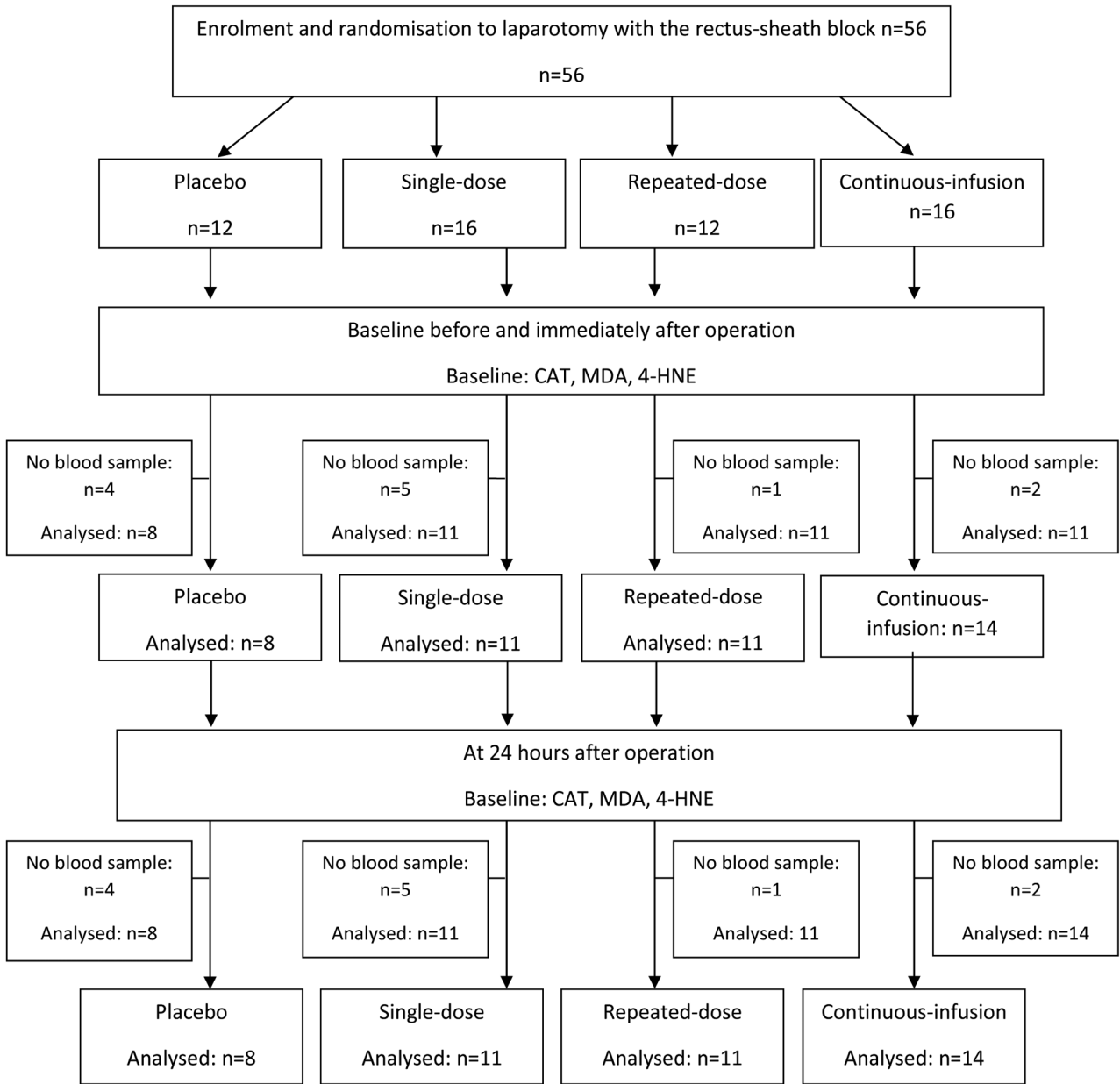


Figure 1. Study flowchart.

laparotomy in patients with benign disease *versus* cancer is unknown. Therefore, the present study investigated the 4-HNE plasma concentrations in patients with benign disease *versus* cancer following midline laparotomy. Our aim was to determine plasma 4-HNE levels in patients with benign disease *versus* patients with cancer and to determine the association between plasma 4-HNE concentrations and the patient satisfaction 24 h following surgery (SFS<sub>24</sub>), as well as the overall pain at rest (NRS<sub>r</sub>) and at 2 kg pressing (NRS<sub>p</sub>).

## Patients and Methods

*Patients in the study.* The study was approved by the Ethics Committee of the Northern Savo Hospital District, Kuopio, Finland (DNRO 120/2011, November 11, 2011) and was registered in the EudraCT database (EudraCT number 2011-005136-25, Consort diagram, Figure 1) and in the ClinicalTrials.gov database (ClinicalTrials.gov Identifier: NCT02869841). The study protocol and inclusion/exclusion criteria of the study patients were detailed in the previous report by Purdy *et al.* (3).

Table I. The clinical data of the four study groups. Data are mean ( $\pm$ standard deviation) or number of cases.

Variable	Control n=8	Single n=11	Repeated n=11	Continuous n=14	<i>p</i> -Value
Age, years	62.6 (14.3)	60.8 (12.6)	63.3 (10.8)	58.0 (10.1)	0.74
Gender female/male	4/4	7/4	9/2	12/2	0.22
Height, cm	166.6 (8.6)	168.4 (7.9)	165.7 (7.2)	164.3 (6.6)	0.62
Weight, kg	78.6 (11.8)	83.7 (12.8)	67.8 (13.7)	68.8 (10.6)	0.007
BMI, kg/m <sup>2</sup>	28.3 (3.8)	29.6 (4.4)	24.6 (4.3)	25.7 (4.9)	0.03
Time in the operative room, min	229.4 (113.4)	274.9 (148.4)	235.7 (112.0)	279.7 (178.5)	0.85
Operative time, min	209.6 (141.2)	221.8 (156.4)	154.4 (95.0)	253.3 (168.9)	0.55
Perioperative-bleed, ml	696 (741)	822(906)	697 (967)	1340(928)	0.31
ASA 1/2/3/4	0/6/2/0	0/7/3/1	0/5/6/0	2/7/5/0	0.43
NRS <sub>r</sub>	2 (1-6)	2 (0-4)	2 (0-3)	1 (0-4)	0.41
NRS <sub>p</sub>	5 (3-9)	5 (1-9)	3.5 (1-7)	4 (0-7)	0.42
SFS <sub>24</sub>	8 (7-8)	9 (8-10)	9.5 (8-10)	10 (8.3-10)	0.001
Length of the skin incision(s), mm	27.2 (6.6)	24.4 (7.8)	24.2 (7.9)	29.7 (7.3)	0.31
Type of disease					0.32
Benign (n=15)	1	3	5	6	
GI cancer (n=9)	3	2	2	2	
Gyn cancer (n=17)	3	4	4	6	
Other cancer (n=3)	1	2	0	0	

GI: Gastrointestinal tract; Gyn: gynaecological; BMI: body mass index; ASA: American Society of Anesthesiologists physical status score; NRS: 11-point numeric rating scale 24 h after surgery; NRS<sub>r</sub>: NRS at rest; NRS<sub>p</sub>: NRS under 2 kg pressure to the wound area; SFS<sub>24</sub>: satisfaction following surgery; RSB: rectal sheath block.

**Plasma biomarkers and outcome measures.** The primary outcome measures were the plasma concentrations of CAT, MDA and 4-HNE biomarkers measured at three time points with high-sensitivity assays: before (PRE), immediately after (POP1) and 24 h postoperatively (POP2) in the control group *versus* three active groups. The patient satisfaction at 24 h postoperatively were filed on a 11-point numeric rating scale (SFS<sub>24</sub>; 0=fully unsatisfied; 10=fully satisfied) and the overall pain at rest (NRS<sub>r</sub>) and at 2 kg pressing (NRS<sub>p</sub>) was surveyed and filed on a 11-point numeric rating scale 24 h following surgery (NRS; 0=no pain; 10=worst pain).

**Samples collection.** EDTA-blood samples were taken at the pre-specified time-points and centrifuged at 1,000 × *g* for 15 min. Plasma was separated and stored frozen at -70°C until further analysis. The plasma CAT assays were performed using enzyme-linked immunosorbent assay ELISA Kit (Cloud-Clone Corporation, Katy, TX, USA). Plasma MDA concentrations were determined by the MDA ELISA Kit (E-EL-0060, Elabscience, Houston, TX, USA). The manufacturer's intra-assay and inter-assay Cvs were 5.6% and 6.28%, respectively. The plasma 4-hydroxynonenal (4-HNE) concentrations were measured at three time points with 4-Hydroxynonenal Elisa kit (Cat# 9705, Alpha Diagnostic Intl., San Antonio, TX, USA): before (PRE), immediately after (POP1) and 24 h postoperatively (POP2). The manufacturer's intra- and inter-assay Cvs were <8% and <10%.

**Statistical analysis.** Data are presented as means and standard deviations or frequencies and percentages, where appropriate. Differences in baseline characteristics between groups were tested by Fisher's exact test and in the case of continuous data, the analysis was performed by Kruskal-Wallis test. Group differences at three time points were tested by the Wilcoxon signed rank test and the Kruskal-

Wallis test. The plasma CAT, MDA and 4-HNE concentrations and differences in the patients with benign diseases and cancer were tested by the Mann-Whitney *U*-test. Linear mixed effect (LME) model was used to test time effect. In LME analysis plasma concentrations were log transformed. The results of the laboratory measurements are presented as median concentrations with interquartile range as distributions were right-skewed. *p*-Values under 0.05 were considered statistically significant. The Spearman's method was used to test for correlation for plasma 4-HNE concentrations *versus* MDA concentrations. Data were analyzed by IBM SPSS statistical software (SPSS Statistics 26.0.0.1; IBM, Armonk, NY, USA).

## Results

**Patient data.** There were 56 patients, of whom 12 were excluded due to missing plasma samples. The final study cohort consisted of 15 patients with benign disease and 29 patients with cancer (Table I). There were significantly less benign patients than cancer patients in the control group (one benign patient *versus* seven cancer patients, benign-cancer ratio=14.3%) than that in the RSB study groups combined (14 benign patients *versus* 22 cancer patients, benign-cancer ratio=63.6%). The RSB analgesia enhanced significantly the median SFS<sub>24</sub> scores in the following order: control, single dose, repeated dose and continuous dose group (8, 9, 9.5 and 10, respectively; *p*=0.001, Table I). There were no statistically significant differences detected in the NRS<sub>r</sub> and NRS<sub>p</sub> scores between the four study groups (NRS<sub>r</sub> *p*=0.41 and NRS<sub>p</sub> *p*=0.42, Table I).

Table II. The plasma catalase (CAT), malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) concentrations in the four study groups. Plasma concentrations were measured before (PRE), immediately after (POP1) and 24 h after (POP2) surgery. Median (interquartile range) concentrations are shown. The Kruskal-Wallis test was used.

Marker	Control	Single	Repeated	Continuous	p-Value
CAT (pg/ml)					<b>0.506</b>
PRE	0.95 (0.67-2.36)	0.74 (0.45-1.58)	0.61 (0.41-1.09)	0.61 (0.43-1.17)	0.407
POP1	1.36 (0.89-3.71)	1.89 (0.78-2.93)	1.53 (0.78-2.17)	1.39 (0.84-2.03)	0.907
POP2	1.17 (0.58-1.65)	0.86 (0.60-1.73)	1.15 (0.77-1.50)	1.06 (0.83-1.92)	0.891
MDA (ng/ml)					<b>0.448</b>
PRE	642 (480-1241)	872 (670-1132)	795 (685-907)	983 (688-1329)	0.478
POP1	541 (321-1165)	753 (576-801)	555 (430-698)	638 (525-909)	0.484
POP2	480 (413-946)	651 (533-836)	577 (343-799)	601 (373-784)	0.767
4-HNE (pg/ml)					<b>0.608</b>
PRE	245 (211-562)	270 (153-327)	264 (209-356)	274 (177-363)	0.979
POP1	207 (164-289)	242 (122-302)	203 (167-409)	177 (106-322)	0.856
POP2	153 (56-187)	132 (75-155)	119 (52-191)	107 (90-206)	0.862

Linear mixed model *p*-values for interaction time group are in bold.

Plasma CAT, MDA and 4-HNE concentrations in the RSB study groups. There were no differences detected in the CAT, MDA and 4-HNE concentrations between the four study groups preoperatively and following surgery (Table II). Although, the patients in the control group had a trend for a slightly higher median CAT levels preoperatively compared to the single dose group, repeated dose and continuous dose groups (0.95 pg/ml versus 0.74, 0.61 and 0.61 pg/ml, respectively, Table II). The patients in the control group had a trend for lower median MDA concentrations preoperatively and following surgery (POP1), compared to RSB study groups (control group, 642/541 ng/ml versus single dose; 872/753 ng/ml and repeated dose; 795/555 ng/ml and continuous infusion; 983/638 ng/ml, Table II). There were no significant differences detected in the preoperative 4-HNE concentrations in RSB study groups and between control groups (Table II). However, the patients in the control group and in the RSB study groups had a trend for lower 4-HNE concentrations following surgery and the most significant postoperative decrease was in patients in the repeated dose and continuous dose groups (Table II).

The alteration of plasma CAT, MDA and 4-HNE levels following surgery. The alteration of plasma CAT, MDA and 4-HNE marker levels in the study groups combined are shown in Table III. There were statistically significant increases in the median plasma concentrations of CAT preoperatively (PRE) and immediately after operation (POP1) (52.5% increase, *p*<0.001, Table III). Then the median plasma concentrations of CAT marker decreased 24 h postoperatively (POP2) (*p*=0.001, Table III). The median plasma concentrations of MDA decreased 24 h postoperatively (POP2) and the decrease was significant (31.6% decrease, *p*<0.001, Table III). There was significant decrease in the

Table III. The alteration of the plasma catalase (CAT), malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) concentrations following surgery in all groups combined (all patients). Plasma concentrations were measured before (PRE), immediately after (POP1) and 24 h postoperatively (POP2). Median (interquartile range) concentrations are shown

Marker	All patients	Alteration	p-Value*
CAT (pg/ml)			
PRE	0.66 (0.48-1.17)		
POP1	1.39 (0.84-2.43)	PRE vs. POP1	<0.001
POP2	1.03 (0.70-1.57)	POP1 vs. POP2	0.001
MDA (ng/ml)			
PRE	870 (625-1118)		
POP1	620 (432-860)	PRE vs. POP1	<0.001
POP2	595 (410-831)	POP1 vs. POP2	0.047
4-HNE (pg/ml)			
PRE	265 (201-348)		
POP1	203 (133-317)	PRE vs. POP1	0.001
POP2	132 (80-175)	POP1 vs. POP2	<0.001

\*Wilcoxon signed-rank test.

median plasma concentrations of MDA preoperatively and immediately after operation (*p*<0.001, Table III). Also, the median plasma concentrations of 4-HNE decreased 24 h following surgery (POP2) and the decrease was statistically significant (50.2% decrease, *p*<0.001, Table III).

Plasma CAT, MDA and 4-HNE concentrations in benign versus cancer patients. The median (interquartile range) plasma concentrations of CAT, MDA and 4-HNE did not differ significantly between the benign and cancer patients preoperatively (Table IV). Patients in the cancer group had a trend for higher median CAT levels postoperatively (POP1)

Table IV. The plasma catalase (CAT), malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) concentrations in the patients with benign diseases (n=15) and in those with cancer (n=29). Plasma concentrations were measured before (PRE), immediately after (POP1) and 24 h postoperatively (POP2). Median (interquartile range) concentrations are shown. Mann-Whitney U-test was used.

Marker	Benign	Cancer	p-Value
CAT (pg/ml)			<b>0.718</b>
PRE	0.62 (0.41-1.17)	0.73 (0.48-1.30)	0.407
POP1	1.09 (0.78-2.50)	1.46 (1.13-2.36)	0.304
POP2	0.83 (0.75-1.65)	1.12 (0.68-1.50)	0.593
MDA (ng/ml)			<b>0.353</b>
PRE	920 (716-1326)	868 (571-1037)	0.211
POP1	852 (614-955)	589 (392-730)	0.021
POP2	697 (407-868)	560 (411-758)	0.383
4-HNE (pg/ml)			<b>0.037</b>
PRE	266 (224-281)	263 (191-358)	0.970
POP1	218 (184-334)	202 (115-310)	0.328
POP2	107 (66-187)	133 (86-174)	0.495

Linear mixed model p-values for interaction time group are in bold.

compared to benign patients (Table IV). There was a statistically significant difference in the median MDA concentrations between benign and cancer patients immediately following surgery (POP1), with the cancer patients showing significantly lower MDA concentrations postoperatively ( $p=0.021$ , Table IV). There was a statistically significant decrease in 4-HNE concentrations postoperatively in benign and cancer patients (Figure 2 and Table IV). In addition, the time effect in the linear mixed model in plasma 4-HNE was statistically significant (Table IV).

*Correlation of SFS<sub>24</sub>, NRS<sub>r</sub> and NRS<sub>p</sub> versus the LP biomarkers MDA and 4-HNE.* NRS<sub>r</sub> correlated significantly to 4-HNE plasma concentrations ( $p=0.026$ ). However, there was no significant correlation between SFS<sub>24</sub> or NRS<sub>p</sub> versus MDA or 4-HNE plasma concentrations. The median (interquartile range) plasma concentrations of MDA correlates significantly to 4-HNE concentrations in benign and cancer patients (Figure 3,  $r=0.347$ ,  $p<0.001$ ).

## Discussion

Albeit the surgical procedure is a common cause of LP in patients with midline laparotomy, the assessment of LP stress biomarker 4-HNE concentrations is rarely reported (1). Especially, the 4-HNE plasma levels versus the patient satisfaction and the NRS pain scores following surgery in midline laparotomy patients is unknown. To cast further light on this issue, the present study was designed to conduct a detailed analysis of the i) Plasma CAT, MDA and 4-HNE concentrations in benign versus cancer patients, ii) The

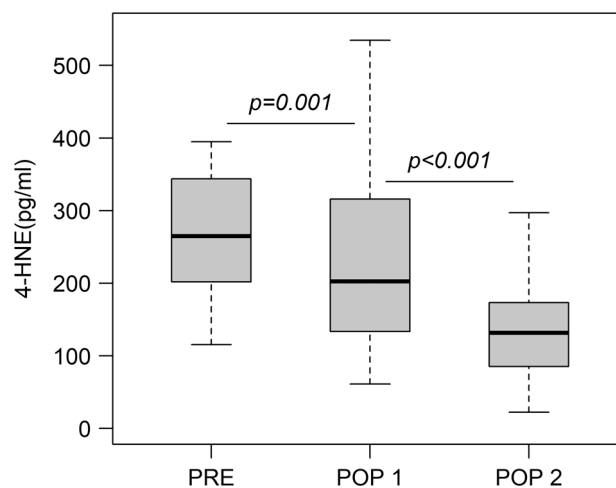


Figure 2. Plasma median 4-hydroxynonenal (4-HNE) concentrations in benign and cancer patients preoperatively, immediately after operation and 24 h after operation.  $p\leq 0.001$  using the Wilcoxon signed-rank test.

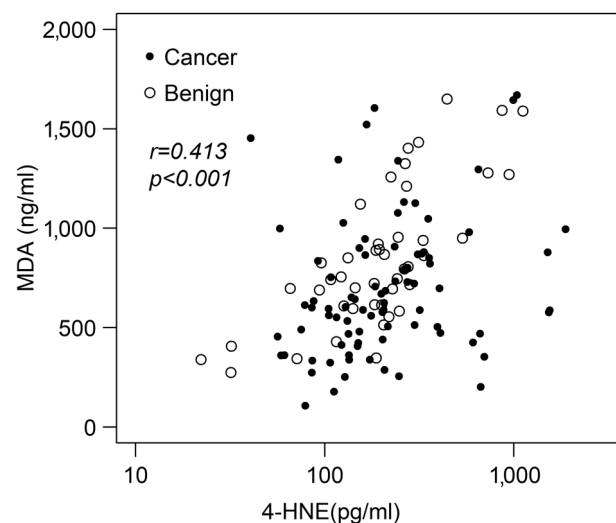


Figure 3. Plasma malondialdehyde (MDA) concentrations versus plasma 4-hydroxynonenal (4-HNE) concentrations in benign and cancer patients shown as Jitterplot. Spearman's  $r=0.413$ ,  $p<0.001$ .

alteration of plasma CAT, MDA and 4-HNE levels in RSB study groups, and iii) Plasma CAT, MDA and 4-HNE concentrations in RSB study groups, to establish whether these plasma biomarkers could improve the detection of OS or LP stress following surgery. The original study hypothesis was that RSB analgesia could lower the pain and enhance satisfaction in midline laparotomy patients. Therefore, the main aim of the study was to assess the relation between plasma 4-HNE concentrations and RSB analgesia in patients with midline laparotomy.



The primary products of LP are lipid hydroperoxides (LOOH) (1, 2). Among the many different aldehydes which can be formed as secondary products during LP, MDA and 4-HNE are receiving most attention because they are being considered as possible risk factor of cancer, cardiovascular diseases, chronic inflammation, diabetes, liver disease, Parkinson's disease, and respiratory distress syndrome (1, 2). In addition, LP products have been suggested as a possible risk factor for Alzheimer's disease (1, 2). In everyday life, attention must be paid to household oils used regularly in caterings and cooking, because in those processes very high amounts of LP products are generated and they can be easily absorbed through the diet (1, 2). The production and plasma level of MDA and 4-HNE can be measured and used as a biomarker to estimate the degree of oxidative stress and lipid peroxidation in human disease (1, 2). Both are toxic substances reacting with deoxyadenosine in DNA and forming DNA adducts, which are mutagenic (1, 2). The aldehydes which can be formed as secondary products during LP have been reviewed by Ayala *et al.* (2), where MDA is shown to be the most mutagenic product of LP, whereas 4-HNE is the most toxic product (2).

One possible bias of the study is that the patients in the control group and in the single-dose group had significantly higher mean body weight and body mass index than patients in the repeated-dose and continuous-infusion group. Unfortunately, we have no explanation for this trend, and due to the small sample size ( $n=44$ ), its significance should be very carefully interpreted. Despite that, following surgery, the RSB study groups did not differ in terms of NRS pain at rest and pain under 2 kg pressure to the wound area. However, the statistically significant inverse correlation between the individual plasma 4-HNE values and NRS pain at rest scores might suggest some role for 4-HNE. In addition, SFS<sub>24</sub> differed significantly between study groups, the repeated-dose (score 9.5) and continuous-infusion group (score 10) patients having highest SFS<sub>24</sub> scores 24 h following surgery.

Although, the NRS<sub>r</sub> correlated significantly to 4-HNE plasma concentrations ( $p=0.026$ ), there was no statistically significant difference shown between plasma 4-HNE concentrations and the NRS<sub>r</sub>/NRS<sub>p</sub> and SFS<sub>24</sub> scores in the control and three RSB study groups. Interestingly, in the present study there was a significant decrease in 4-HNE concentrations following surgery in benign and cancer patients. In addition, the time effect in the linear mixed model in plasma 4-HNE was statistically significant. Saimanen *et al.* (12, 13) reported earlier that RSB-analgesia significantly increases the CAT concentrations and decreases the NT concentrations (20) immediately after operation. Surprisingly CAT concentrations dropped back 24 h postoperatively. Kärkkäinen *et al.* (15) reported earlier that RSB-analgesia does significantly increase the SOD-1 concentrations immediately after operation, but SOD-1 concentration drops back 24 h postoperatively. Martins *et al.* (21) have previously demonstrated that accumulation of

4-HNE plays a major role in the establishment and progression of pain showing that the 4-HNE is an endogenous aldehyde generated during oxidative stress, mainly through the LP process in the mitochondria. Therefore, increased 4-HNE adducts in the injured tissue positively correlates with pain (21). Sauer *et al.* (22) showed that the Delphinidin (DEL), a plant-derived antioxidant with potential to treat inflammatory pain, prevented 4-HNE-induced mechanical hyperalgesia, cold allodynia, and an increase in the intracellular calcium concentration into transient receptor potential ankyrin 1 expressing cells. The aim of their study was to develop a well-tolerated cyclodextrin (CD)-DEL complex with enhanced bioavailability and to investigate the mechanisms behind its antinociceptive effects in a preclinical model of inflammatory pain, and formation of the LP product 4-HNE.

## Conclusion

The enhanced ROS production alters the mitochondrial genome and proteome function through the accumulation of LP products, such as 4-HNE and MDA. Some mitochondrial protein products (*e.g.*, Sirtuin3, SIRT3) can reduce ROS levels by modulating key antioxidant enzymes, such as manganese superoxide dismutase (MnSOD) (23). The present study showed that plasma 4-HNE concentrations decreased significantly after operation in all patients and 4-HNE concentrations were inversely correlated to the overall pain at rest (the NRS<sub>r</sub> pain scores). The present study confirms the applicability of the plasma biomarker 4-HNE to cast further light on the postoperative pain in midline laparotomy patients.

## Conflicts of Interest

The Authors report no conflicts of interest or have any financial ties to disclose.

## Authors' Contributions

The Authors M.E., I.S., R.K., A.H., T.S. and M.E. did the collection and analysis of data. M.E., I.S., R.K., A.H., T.S. and M.E. drafted and revised the manuscript, read and approved the final article.

## Acknowledgements

The study was funded by the Heikki, Aino and Aarne Korhonen Foundation, Kuopio, Finland, and the Päivikki and Sakari Sohlberg Foundation, Helsinki, Finland.

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Received January 21, 2022

Revised February 8, 2022

Accepted February 15, 2022