ELECTRONIC SUPPLEMENTARY MATERIAL

Krieg N *et al.*: Prognostic value of perioperative changes in serum primary metabolites in patients after major surgery under general anesthesia: an exploratory secondary analysis of the TAPIR trial

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eAppendix 1 Definition of high-risk patients

Patients were eligible for inclusion if they were 18 years of age or older and were scheduled to undergo major abdominal surgery (general, urological, gynaecological, or vascular surgery) with an expected duration of at least 90 minutes or an expected blood loss of at least 1,000 ml.[1]

The presence of at least one of the following high-risk criteria was required:[1]

- acute or chronic renal impairment (serum creatinine ≥1.3 mg/dL)
- predefined risk factor for cardiac or respiratory complications (i.e., exercise tolerance equivalent to four metabolic equivalents [METs] or less as defined by American College of Cardiology/American Heart Association guidelines
- past medical history of ischemic heart disease [angina, myocardial infarction or acute coronary syndrome]
- angiographically proven ischemic heart disease
- ejection fraction less than 30% in echocardiography
- moderate or severe valvular heart disease, history or clinical signs of chronic heart failure [e.g., requiring treatment, oedema]
- clinical history of chronic obstructive pulmonary disease [COPD]
  - poor lung function demonstrated by spirometry [forced expiratory volume in 1 second (FEV1) or forced vital capacity (FVC) <75% predicted]
- radiographically confirmed chronic lung disease [e.g., fibrosis, COPD]
- immunodeficiency due to a therapy [e.g., immunosuppressants, chemotherapy, radiation, long-term or high-dose steroids]
- immunodeficiency due to specific diseases [e.g., leukaemia, lymphoma, AIDS]
- severe liver impairment [biopsy proven liver cirrhosis plus one of the following: portal hypertension or history of upper gastrointestinal bleeding due to portal hypertension or previous episodes of hepatic insufficiency/hepatic encephalopathy/hepatic coma]
- or age of equal to or higher than 80 years

Detailed definition of risk factors for cardiac or respiratory complications
Patients with risk factors for cardiac or respiratory complications had to meet at least one of the following criteria:[1]

- exercise tolerance equivalent to four metabolic equivalents or less as defined by American College of Cardiology/American Heart Association guidelines
- past medical history of ischemic heart disease (angina, myocardial infarction or acute coronary syndrome)
- angiographically proven ischemic heart disease
- ejection fraction less than 30% in echocardiography
- moderate or severe valvular heart disease
- history or clinical signs of chronic heart failure (e.g. requiring treatment, oedema)
- clinical history of chronic obstructive pulmonary disease (COPD)
- poor lung function demonstrated by spirometry (FEV1 or FVC < 75% predicted)
- radiographically confirmed chronic lung disease (e.g. fibrosis, COPD)

eAppendix 2 Postoperative complications

Frequencies of postoperative complications. Complications were obtained according to the European Perioperative Clinical Outcome definitions.[1,2]

	Total (*N* = 177)	
Composite of predefined major postoperative complications and mortality at day 30 after surgery, n/total N (%)	75/177	(42.4%)
Single outcome measures[1]		
Mortality, n/total N (%)	4/176	(2.3%)
Myocardial ischemia or infarction, n/total N (%)	0/176	(0.0%)
Arrhythmia, n/total N (%)	7/176	(4.0%)
Cardiac arrest, n/total N (%)	3/176	(1.7%)
Limb ischemia, n/total N (%)	1/176	(0.6%)
Cardiogenic pulmonary oedema, n/total N (%)	1/176	(0.6%)
Deep vein thrombosis, n/total N (%)	0/176	(0.0%)
Pulmonary embolism, n/total N (%)	2/176	(1.1%)
Acute respiratory distress syndrome, n/total N (%)	1/176	(0.6%)
Gastrointestinal bleeding, n/total N (%)	1/176	(0.6%)
Bowel infarction, n/total N (%)	0/176	(0.0%)
Anastomotic breakdown, n/total N (%)	7/176	(4.0%)
Paralytic ileus, n/total N (%)	2/175	(1.1%)
Acute kidney injury[2], n/total N (%)	15/177	(8.5%)
Infection, source uncertain, n/total N (%)	5/177	(2.8%)
Delirium, n/total N (%)	13/177	(7.3%)
Urinary tract infection, n/total N (%)	8/177	(4.5%)
Surgical site infection, n/total N (%)	36/177	(20.3%)
Laboratory-confirmed blood stream infection, n/total N (%)	11/177	(6.2%)
Hospital-acquired pneumonia, n/total N (%)	18/177	(10.2%)
Postoperative haemorrhage, n/total N (%)	3/176	(1.7%)
Stroke, n/total N (%)	2/176	(1.1%)
Infection, composite score, n/total N (%)[3]	66/177	(37.3%)

[1]Some patients developed more than one complication
[2] Kidney Disease Improving Global Outcomes guidelines Stage 1 or higher (Stage 1: serum creatinine 1.5-1.9 times baseline value within 7 days or ≥27 μmol L^{-1} (0.3 mg dl^{-1}) increase within 48 h), [3] composite of infection (source uncertain), urinary tract infection, surgical site infection, laboratory-confirmed blood stream infection, and hospital-acquired pneumonia

eAppendix 3 Metabolomics analysis

3.1 Sample processing and experimental equipment

The blood draws included a blood sample, if possible, from existing lines, such as a central venous catheter or artery necessary for monitoring. Serum tubes were kept upright at room temperature for 120 minutes and then centrifuged for 15 minutes at 3,000 RPM without braking. The supernatant was aliquoted in quantities of 500 µl each into 1.5 ml Eppendorf tubes and frozen at -20 °C. 20 µL of serum sample was precipitated with 200 µL of LCMS-grade methanol. Prior to processing, the methanol was spiked with internal standard (IS) 2-morpholinoethanesulfonic acid (Sigma-Aldrich, Darmstadt, Germany) (i.e. 10 nM final concentration). The supernatant was taken for analysis after 4 days of incubation at −80 °C. After incubation at −80 °C centrifugation was performed at 2,272 RCF for 20 minutes to obtain the supernatant. Metabolites were analysed by liquid chromatography coupled with triple quadrupole mass spectrometry (LC-MS/MS) using the high-performance liquid chromatography (HPLC) system (Prominence series) with an inert kit and the triple quadrupole mass spectrometer LCMS-8050 from Shimadzu Deutschland GmbH (Duisburg, Germany). The volume of the sample injection was set to 10 µL. Primary metabolites were analysed using the HPLC column Discovery® HS F5, 3 µm, 150 mm x 2,1 mm from Sigma-Aldrich Chemie GmbH (Munich, Germany). Mobile phase A and B consisted of 0.1% formic acid in water and of 0.1 % formic acid in acetonitrile, respectively. Mass spectrometric detection was performed by multiple reactions monitoring (MRM). Serum samples were analysed with the supplied method package 'primary metabolites' according to the manufacturer's protocols. Primary data were analysed and further processed with LabSolutions 5.91 and LabSolutions Insight 3.10 from Shimadzu Deutschland (Duisburg, Germany).

3.2 LC-MS settings

HPLC program. Targeted analytes were eluted using a programmed gradient of the eluents 0.1 % formic acid in water and 0.1 % formic acid in acetonitrile (solvent B) at a flow rate of 0.25 ml/min and an oven temperature of 50 °C.

time	**command**	**value**
[minutes]		[percent]
00.01	solvent B concentration	0
02.00	solvent B concentration	0
05.00	solvent B concentration	25
11.00	solvent B concentration	35
15.00	solvent B concentration	95
20.00	solvent B concentration	95
20.01	solvent B concentration	0
25.00	stop	

Mass spectrometer settings (LCMS 8050)

source conditions	parameters
nebulizing gas flow rate	3.0 L·min^{-1}
heating gas flow rate	10.0 L·min^{-1}
drying gas flow rate	10.0 L·min^{-1}
collision-induced dissociation gas pressure	230 kPa
interface temperature	300 °C
desolvation line temperature	250 °C
block heater temperature	400 °C
ionization mode	electrospray ionisation (ESI)

3.3 Mass transitions

Mass transitions. The target ion shows the multiple reaction monitoring (MRM) transitions, the ionization polarity (IP) shows the ionization mode of the ESI source and the internal standard (IS) column assigns the number of the internal standard to the compounds with which they were evaluated. The internal standard is marked bold.

no.	compound	target ion	IP	IS	KEGG[1]
1	4-hydroxyproline	132.10>86.05	+	1	C01157
2	adenosine	268.10>136.05	+	1	C00212
3	alanine	89.90>44.10	+	1	C00041
4	asparagine	133.10>87.15	+	1	C00152
5	cholesterol	369.4>161.30	+	1	C00187
6	citrulline	176.10>70.05	+	1	C00327
7	creatinine	114.10>44.05	+	1	C00791
8	cystine	241.00>151.95	+	1	C00491
9	deoxycholic acid/chenodeoxycholic acid	391.30>391.30	-	1	C04483/ C02528
10	dimethylarginine (symmetric/asymmetric)$^{2+}$	203.1>70.15	+	1	C21189/ C21188
11	dimethylglycine	104.10>58.05	+	1	C01026
12	glutamic acid	147.90>84.10	+	1	C00025
13	glutamine	147.10>84.15	+	1	C00064
14	glycine	75.90>30.15	+	1	C00037
15	glycodeoxycholic acid	448.3>74.20	-	1	C05464
16	guanosine	284.00>152.00	+	1	C00387
17	histidine	155.90>110.10	+	1	C00135
18	hypoxanthine	137.00>55.05	+	1	C00262
19	inosine	269.10>137.05	+	1	C00294
20	isoleucine	132.10>86.20	+	1	C00407
21	leucine	132.10>86.05	+	1	C00123
22	methionine	149.90>56.10	+	1	C00073
23	methionine sulfoxide	166.00>74.10	+	1	
24	niacinamide	123.10>80.05	+	1	C00153

no.	compound	target ion	IP	IS	KEGG[1]
25	pyruvic acid	86.90>87.05	-	1	C00022
26	serine	105.90>60.10	+	1	C00065
27	succinic acid	117.30>73.00	-	1	C00042
28	taurochenodeoxycholic acid	498,40>498.40	-	1	C05465
29	taurocholic acid	514.20>514.10	-	1	C05122
30	threonine	120.10>74.15	+	1	C00188
31	thymidine monophosphate	322.90>81.10	+	1	C00364
32	tryptophan	205.10>188.15	+	1	C00078
33	tyrosine	182.10>136.10	+	1	C00082
34	uric acid	167.10>123.95	-	1	C00366
35	uridine	245.00>113.05	+	1	C00299
36	valine	118.10>72.15	+	1	C00183
37	xanthine	151.00>108.00	-	1	C00385
38	**2-morpholinoethanesulfonic acid (IS)**	194.00>80.15	-	1	

[1]KEGG = Kyoto Encyclopedia of Genes and Genomes (freely available electronic database with information about genomes, biological pathways, diseases, drugs, and chemical substances for bioinformatic analysis)
[2] LC-MS/MS measurements do not distinguish between isomers

eAppendix 4 Additional subgroup analysis

Age, sex and comorbidities may have an additional impact on metabolism, leading to inter-individual variability in the effects of surgery under general anesthesia on the metabolism. To investigate age-, sex-, and comorbidity-specific metabolic alterations, we analysed pre- and postoperative differences in levels of primary serum metabolites in patients ≥ 65 years (*vs* younger), male (*vs* female) patients, patients with (*vs* without) chronic kidney disease (CKD, serum creatinine at admission ≥ 1.3 $mg \cdot dL^{-1}$ *vs* below), patients with obesity (BMI ≥30 $kg \cdot m^{-2}$ *vs* below), patients with immunosuppression (*vs* without), and patients with risk factors for cardiac or respiratory complications (*vs* without; definition in ESM eAppendix 1). Statistical analysis was performed according to the subgroup analysis of postoperative complications (see main manuscript).

4.1 Results

4.1.1 Preoperative differences

We found no differences in serum metabolites for patients with obesity (BMI ≥30 $kg \cdot m^{-2}$ *vs* below) and patients with (*vs* without) immunosuppressive therapy (all false-discovery rate [FDR] adjusted P values > 0.05, eFigure).

Male (*vs* female) patients showed lower levels of guanosine. Patients ≥ 65 years (*vs* younger) showed significantly lower levels of 4-hydroxyproline. In patients with (*vs* without) chronic kidney disease, levels of creatinine were significantly higher. Patients with (*vs* without) cardiac/respiratory risk factors showed significantly lower levels of 4-hydroxyproline, glutamine, glycine, guanosine, histidine, inosine, and niacinamide (details in ESM eTable 3).

4.1.2 Postoperative differences

We found no significant differences for patients with (*vs* without) obesity or immunosuppressive therapy (all FDR adjusted P values >0.05). Male (*vs* female) patients showed higher levels of creatinine (eFigure). Older (*vs* younger) patients showed lower levels of 4-hydroxyproline. Patients with chronic kidney disease showed higher levels of creatinine and methionine sulfoxide. In patients with (*vs* without) cardiac/respiratory risk factors, we found lower levels in eleven metabolites. However, after adjusting for preoperative metabolite levels none of the aforementioned differences reached significance (all FDR adjusted P values >0.05), except for lower levels of hypoxanthine, isoleucine, leucine, serine, and valine in patients with (*vs* without) cardiac/respiratory risk factors (details in ESM eTable 3). None of the interaction terms (*Group* × *Time*) showed FDR adjusted P values <0.05.

eFigure Heatmap for the main findings of the explorative analyses on subgroups. We present pre- (D0) and postoperative (D3) model based mean differences (unit: z-score). In addition we present postoperative mean differences adjusted for preoperative metabolite levels (ANCOVA: D3). Negative/positive values indicate lower/higher serum metabolite levels in the group indicated in the overarching columns (e.g. higher/lower values for male *vs* female patients). In addition, we report the regression weight of the interaction term of *Group* and *Time* (Group × Time), i.e. difference in change between D0 and D3 between the groups. Per subgroup analysis (overarching columns), all P values (D0, D3, D3: ANCOVA, and Group × Time) were adjusted using the false-discovery rate (FDR) approach. The serum metabolites were grouped according to hierarchical cluster analysis (Euclidean distances of the correlation matrix of metabolite levels at D0, agglomerative clustering with complete linkage). [* *P* value of model based mean difference/ regression weight <0.05; ** *P* value <0.05 after FDR adjustment for multiple comparisons; interpretation of the absolute values of the mean differences/regression weights: >0.1 small, >0.3 medium and >0.5 large effect size]. DCA/CDCA: deoxycholic acid/chenodeoxycholic acid

4.2 Discussion

Sex. Preoperatively male patients showed lower levels of guanosine (eFigure). The nucleoside guanosine undergoes reutilization or degradation to uric acid. Publications on sex-specific guanosine levels or levels of related derivates like guanosine monophosphate (GMP) are rare and distributed over a wide range of topics: circulating GMP in Malay population, guanosine distribution in the brain.[3, 4] These publications along with our results seem to support preoperative sex-specific guanosine levels.

Age. Patients of older age (≥ 65 years *vs* younger) showed lower 4-hydroxyproline levels. 4-hydroxyproline can be metabolized to glyoxylate, pyruvate or glycine and is a major resource of collagen, which constitutes one third of the total protein.[5] Age-related urinary hydroxyproline/creatine ratio is well-documented, but is controversially discussed especially with regard to the dependency of hydroxyproline levels on sex, hormonal levels and acute intake of collagenous protein (< 6 hours).[6-10] In contrast, the role of circulation hydroxyproline is less well studied with different results regarding age.[11, 12] Nevertheless, the biochemical meaning of pre- and postoperative significance has to be further evaluated, because its relevance in aging is unknown.

Chronic kidney disease. In patients with chronic kidney disease, preoperative creatinine levels were expectedly higher due to reduced renal clearance.

C*ardiac and respiratory risk factors.* Significantly lower levels of glutamine, glycine, histidine and inosine were identified preoperatively in patients with (vs without) cardiac and respiratory risk factors. These metabolites play crucial roles in immunomodulation, cardio- and neuroprotection, cellular metabolism, and antioxidant defence. Glycine has demonstrated a protective effect on coronary heart disease[13] and links to a lower myocardial infarction incidence,[14] as well as associations with metabolic syndrome and insulin resistance.[15] Glutamine, a conditionally essential amino acid abundant in healthy states, was depleted in a third of intensive care unit (ICU) patients, particularly those with prolonged critical illness.[16] It plays vital roles in modulating the inflammatory response and immune cell proliferation.[17] Clinical trials of glutamine supplementation in colorectal cancer patients or critically ill surgical patients have shown promising results in reducing infection rates, ICU mortality, and hospital stay.[16, 18] Histidine, an essential amino acid, contributes to immune function and was found to be reduced in patients with chronic respiratory disease.[19] Inosine, a nucleoside derived from adenosine breakdown, exhibits vasodilatory, inotropic, and cardioprotective effects, potentially predicting cardiovascular disease.[20, 21] Furthermore, the levels of niacinamide, 4-hydroxyproline and guanosine were only reduced before surgery. Niacinamide (nicotinamide) is a precursor for niacinamide adenine nucleotide (NAD^+), which acts as a cofactor in various metabolic pathways, cellular energy and redox metabolisms or it can be consumed for DNA repair and mitochondrial signaling.[22, 23] Several research groups reviewed that NAD^+ is involved in key processes of cardiovascular diseases.[24-26] In line with our results, Jing *et al.* described downregulated levels of niacinamide in patients with cardiovascular artery disease in comparison to healthy people.[27] This could be further evidence that niacinamide plays a role in NAD^+ homeostasis in cardiovascular diseases. In addition, altered 4-hydroxyproline levels appear to be linked to cardiovascular disease pathogenesis, including calcific aortic stenosis, acute coronary syndrome,

coronary artery disease and heart failure.[27-31] 4-hydroxyproline is an important component of the *collagen* network that contributes to the contractility of the heart and artery mechanics.[32] Studies, which also found lowered 4-hydroxyproline levels, suggested a ventricular remodeling due to low collagen synthesis in their analyzed cardiovascular risk group.[29, 33] We were unable to identify any study that explicitly investigated the relationship between cardiovascular or respiratory comorbidities and guanosines.

Postoperatively, we found significantly lower levels of branched-chained amino-acids (BCAA; isoleucine, leucine and valine), serine and hypoxanthine. The link between circulating BCAAs and cardiovascular dysfunction has been the subject of both support and contradiction in numerous studies. For instance, increased levels of BCAAs in patients with heart failure and coronary artery disease were associated with subsequent adverse events.[34, 35] In contrast, Szpetnar *et al.* described decreased BCAA levels after myocardial infarction.[36] And a study even described that increased plasma BCAA levels at baseline of patients with heart failure with preserved ejection fraction undergoing cardiac resynchronization therapy were associated with improvement in left ventricular systolic function.[36, 37] Serine contributes to protein synthesis, neurotransmission and various important pathways like the glycerophospholipid and sphingolipid metabolism.[38] Previous studies described a high correlation with different cardiovascular diseases.[29, 39, 40] Therefore, the cardio-protective effects of serine could be attributed to its antihypertensive, anti-inflammatory and anti-oxidant properties.[41-43] Multiple studies have reported a degradation of hypoxanthine levels in patients with heart failure and myocardial injury, which goes in line with findings in our study.[44, 45] Hypoxanthine is an intermediate of the purine metabolism and is metabolized to uric acid, which can act as an antioxidant and is linked to myocardial injury.[46] The postoperatively altered levels of BCAA, serine and hypoxanthine could be related to surgery under-general anesthesia, but medication (e.g. xanthine oxidase inhibitors) could also be partially involved.[47-50]

Conclusion. Preoperatively, significant metabolic differences were evident in male (*vs* female) and older (≥65 *vs* younger) patients and patients with chronic kidney disease and those with cardiac and respiratory risk factors, which are at least in part in line with the current literature. Based on the interpretation of the regression models and considering preoperative metabolite levels, we found no specific perioperative alterations in the analysed patient subgroups. In summary, this indicates that in most metabolites similar changes are caused in the patient subgroups and/or that preoperative differences are enhanced by surgery under general anesthesia.

eAppendix 5 Early postoperative metabolome signatures of postoperative delirium (detailed discussion)

The most significant findings, however, were observed in patients who developed postoperative delirium. Lower levels of serum serine and tyrosine, both known for their role in neurotransmitter generation, were associated with delirium. To our knowledge, this is the first metabolomics analysis that shows that lower levels of serum serine and tyrosine correlates with postoperative delirium after major surgery under general anesthesia. Serine is essential for normal cellular functions and crucial in forming glycine, cysteine, and phospholipids.[51] It exerts neuromodulating functions in the brain through its enantiomers, L-serine and D-serine (which are not individually determined by our instrumentation), as well as its metabolite, glycine.[38] L-serine shows neuroprotective effects, activating glycine receptors to alleviate glutamate neurotoxicity.[52] D-serine acts as a co-agonist for the N-methyl-D-aspartate (NMDA) receptor, playing a role in neurotransmission, learning, and memory.[52] Lower serum serine levels have been linked to depression, schizophrenia, and all-cause mortality in arterial hypertension patients.[53] A low concentration of serum serine may affect the circulating level of these neurotransmitters and could, in turn, play a role in the development of postoperative delirium. In our study, patients affected by postoperative delirium also displayed low levels of tyrosine, a large neutral amino acid and a precursor for the neurotransmitters dopamine and noradrenaline.[54] This finding suggests that the decrease in tyrosine may be linked to increased consumption for enhanced dopamine production or reduced availability. Although increased dopamine levels have been found in patients with hyperactive delirium,[55] treatment with dopamine receptor blockers like haloperidol and risperidone failed to show any significant clinical improvement,[56,57] indicating a more complex underlying pathophysiology.

In recent years, changes in citrulline levels have been analyzed in patients with delirium and postoperative delirium, with varying results.[58-61] Studies that showed a delirium-relevant connection suggested a dysregulation of citrulline in its role as arginine regulator and nitrogen producer, which results in uncontrolled oxidative damage.[58, 60, 62, 63] In addition, alanine, aspargine, cystine, glutamic acid, glutamine, and threonine were decreased postoperatively in patients with postoperative delirium in our study. Glutamic acid, an excitatory neurotransmitter in the brain, plays a crucial role in the development of neurodegenerative disorders, e.g. Alzheimer's disease.[64] A correlation has been identified between both, lower preoperative serum glutamine levels[65] and a postoperative decrease in glutamine and threonine levels in elderly patients developing postoperative delirium.[66] This suggests that glutamic acid-glutamine cycle dysfunction may be a contributing factor. For the remaining metabolites, to the best of our knowledge, no specific links to the pathogenesis of delirium have been published.

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