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Ruxolitinib treatment reduces monocytic superoxide radical formation without affecting hydrogen peroxide formation or systemic oxidative nucleoside damage in myelofibrosis

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ABSTRACT

The role of excess reactive oxygen species (ROS) with consequent DNA/RNA damage is now recognized as a hallmark of cancer. In JAK2V617F mutated myeloproliferative neoplasms, ROS have been suggested to be important factors in disease initiation and progression. Ruxolitinib is the most widely used drug for myelofibrosis, because it improves symptom-score. However, both the anti-clonal potential and improvement in overall survival are limited. We investigated the impact of ruxolitinib on formation of superoxide radical and hydrogen peroxide by monocytes in sequentially acquired blood samples from patients with myelofibrosis. We also investigated the impact on RNA and DNA damage by measuring urinary excretion of 8-oxo-Guo and 8-oxo-d-Guo. The formation of superoxide by monocytes was reduced significantly during ruxolitinib therapy, but no impact on the formation of hydrogen peroxide by monocytes or the systemic amount of oxidatively damaged RNA or DNA could be demonstrated. We conclude that ruxolitinib holds little anti-oxidative potential.

Background

In recent years, evidence has accumulated that chronic inflammation may have an important role for the development of the Philadelphia-chromosome negative myeloproliferative neoplasms (MPNs), being the driving force for clonal evolution within the biological continuum from the early cancer stages (essential thrombocythemia (ET) and polycythemia vera (PV)) toward the advanced stages with myelofibrosis (MF) and acute myeloid leukemia (AML) [1–6]. Thus, high-sensitivity C-reactive protein (CRP) is elevated in several patients with ET and PV [7], and circulating levels of proinflammatory cytokines are elevated in most patients, being highly excessive in advanced MF [8–10]. Furthermore, whole blood gene expression studies have shown inflammation and immune genes to be markedly upregulated [11,12].

Oxidative stress (OS) characterizes many different clinical conditions including both benign inflammatory diseases as well as malignant neoplasia [4,11,13–18]. Deregulated cellular metabolism and chronic inflammation are both included in the updated ‘Hallmarks of Cancer’ as emerging hallmarks and enabling characteristics, respectively, and considered important factors in cancer development and progression [19–21]. In most solid cancers, these hallmarks, conceptualized and reviewed by Hanahan & Weinberg [19,20], are thought to be sequentially acquired through different mutations affecting distinct pathways and cellular mechanisms.

Recently, the serum concentrations of reactive oxygen species (ROS) have been found to be increased and the anti-oxidant capacity to be decreased in patients with post-PV-MF and primary MF as compared to healthy individuals [13]. Importantly, the JAK2 mutation per se was associated with a more pronounced redox imbalance than displayed by JAK2-negative MF patients [13]. It has also been demonstrated, that the JAK2 mutation per se induces increased ROS concentration, mainly by deregulating
catalase (which converts reactive H$_2$O$_2$ to harmless H$_2$O and O$_2$). The mutation is associated with an excess of oxidatively damaged RNA and double-stranded DNA breaks causing genomic instability [16]. Furthermore, transplanting mice with a mix of JAK2-mutated and JAK2 wild-type hematopoietic stem cells (HSC) induces a PV phenotype, the development of which can be halted by administration of anti-oxidants (N-acetyl-Cystein, NAC). NAC treatment of mice also reduces the amount of DNA damage, as assessed by a reduction in 8-oxo-guanines and double-stranded DNA breaks (DSBs) [16]. The proposed role of excess ROS in MPNs is also supported by the finding that germline mutations in CHEK2 are common in both ET and PV [22,23]. In healthy cells, the excess amount of ROS and ROS induced DNA damage activate danger-signals inducing either apoptosis (via p53) or cell-cycle arrest in order to allow sufficient DNA-repair. Either way, proliferation of healthy cells is limited in this toxic milieu with excess ROS which gives the clonal proliferation of healthy cells is limited in this toxic milieu with excess ROS which gives the clonal advantage to the clonal JAK2V617F cells a competitive and proliferative advantage thus facilitating clonal expansion [24–26]. Together, these findings suggest that the MPNs may evolve and progress as a result of excess formation of ROS, with consequent genomic instability and clonal expansion as consequence. They also suggest that one single mutation, JAK2V617F, interferes with the cellular machinery in such a fundamental way that several hallmarks of cancer are acquired by one mutation.

Ruxolitinib has demonstrated little anti-clonal potential, and is also associated with dose-limiting hematological adverse events, in particular thrombocytopenia. Ruxolitinib has been associated with an improvement in overall survival but this notion is based on studies that were not designed to investigate this endpoint (e.g. the COMFORT trial had a cross-over design [27]), so further studies are needed to clarify if ruxolitinib indeed possesses such potential [28]. The limited disease-modifying effect of ruxolitinib makes monotherapy less tempting. However the anti-inflammatory potential combined with the potent impact on symptoms make ruxolitinib a promising drug in the adjuvant setting as supplement to drugs that do possess anti-clonal potential [29,30]. The impact of ruxolitinib on the metabolic changes in myelofibrosis and the OS associated with this neoplasm has not been investigated. In light of the above mentioned role of ROS in MPN disease initiation and progression [31], any such potential could prove important when designing future studies. In order to investigate if ruxolitinib has any anti-oxidative potential, we assessed ROS production and oxidatively DNA/RNA damage in a cohort of myelofibrosis patients before and during treatment with ruxolitinib.

Materials and methods

Patients

This study included 35 patients with either post-ET (n = 3), post-PV (n = 10) or primary myelofibrosis (n = 22), who were treated with ruxolitinib. The majority were JAK2 mutated (n = 27), few (n = 2) were verified as being CAL-R mutated but not all patients had the analysis done since testing for CAL-R was not available at the time of inclusion (the study started in 2012 before the mutation was described). Consequently, patients without a designated clonal marker (n = 7) are a mix of CAL-R, MPL, or triple-negative patients. At baseline patients were slightly anemic with an average hemoglobin concentration of 6.69 mM, an average leucocyte-count of 13.05 x 10$^6$/L and an average platelet-count of 273 x 10$^9$/L. See Table 1 displaying baseline characteristics. The only inclusion criteria were initiation of ruxolitinib treatment and the acceptance to participate in the study. Inclusion in the study had no influence on treatment strategy. As such, this was an observational study including biological material for basic research. Some of the patients received ruxolitinib despite low platelet-counts due to lack of other available therapies in this patient category [32]. Dosing was decided by the designated physician during visits in the out-patient clinic. Ruxolitinib dose ranged from 0 to 50 mg daily and was based on clinical and hematological response. Patients were included from the Department of Hematology, Region Zealand University, Roskilde Hospital. They signed an informed consent in accordance with the declaration of Helsinki, and they were diagnosed according to WHO-2008 criteria.

<table>
<thead>
<tr>
<th>Baseline Characteristics</th>
<th>Post ET-MF</th>
<th>Post PV-MF</th>
<th>Primary MF</th>
<th>Total</th>
</tr>
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<tbody>
<tr>
<td>Number</td>
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<td>10</td>
<td>22</td>
<td>35</td>
</tr>
<tr>
<td>Age (years)</td>
<td>63</td>
<td>68.5</td>
<td>71.9</td>
<td>70.2</td>
</tr>
<tr>
<td>Gender (M vs. F)</td>
<td>7 vs. 3</td>
<td>12 vs. 10</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>Driver mutation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JAK2</td>
<td>2</td>
<td>10</td>
<td>14</td>
<td>26</td>
</tr>
<tr>
<td>CALR</td>
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<td>2</td>
<td>2</td>
<td></td>
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<tr>
<td>*Unknown</td>
<td>1</td>
<td>0</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>Periferal blood counts</td>
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<td></td>
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<tr>
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<td>6.7</td>
<td>6.7</td>
<td>6.69</td>
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<td>Leukocytes x 10$^6$/mL</td>
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<td>11.3</td>
<td>15.0</td>
<td>13.05</td>
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<tr>
<td>Platelets x 10$^9$/mL</td>
<td>409.3</td>
<td>346.8</td>
<td>222.0</td>
<td>273.7</td>
</tr>
</tbody>
</table>

*Not all have been tested for CALR, none have been tested for MPL
**Biological samples**

Samples were collected at baseline and subsequently at 1, 3, 6, 9, 12, 18, 24, 30, and 36 months. Patients who were not newly diagnosed were intended to have a ‘wash-out’ period of 1 week without cytoreductive therapy (prior therapies included primarily hydroxyurea but also interferon and HDAC inhibitors), before starting ruxolitinib but this was not always feasible.

**Cells**

Cell counts were determined locally at Roskilde Hospital at the Department of Clinical Biochemistry, by accredited methods.

Venous blood samples were collected in 9 mL lithium-heparin tubes (BD Bioscience, Brøndby, Denmark) and peripheral blood mononuclear cells (PBMCs) were purified using Lymphoprep™ (Axis-Shield, Oslo, Norway) and gradient centrifugation according to manufacturer’s instructions, with the modification that a centrifugal force of 1000 g was used. Upon purification, cells were washed twice in RPMI medium (RPMI 1640, BioWhittaker, Verviers, Belgium) and counted, before suspended in freeze-medium containing RPMI (60%), fetal calf serum (FCS, 30%, Sigma-Aldrich, St. Louis, MO) and di-methyl-sulf-oxide (DMSO, 10%; Merck, Darmstadt, Germany) at a cellular concentration of $10^6$ cells/mL, each vial containing 1 mL. Cells were initially frozen in a Cool-Cell® container at $-80^\circ$C for 2–3 days before final storage in liquid nitrogen until use.

**Cellular assay for ROS-production**

PBMCs collected at baseline and subsequent time points were thawed simultaneously at room temperature, suspended in separate tubes containing 10 mL RPMI (with 5% FCS), and spun at 300 g and 24 $^\circ$C for 5 min. The pellet was re-suspended in 10 mL RPMI. Ten microliter of the cell suspension were stained for viability using Trypan-blue and a light-microscope. Following cell counting, $4 \times 10^6$ viable cells from each sample were spun again and re-suspended in 1 mL pooled serum from healthy blood group AB-positive male donors, (henceforward referred to as ‘AB serum’; Sigma-Aldrich, St. Louis, MO). The cells were stimulated with phorbol 12-myristate 13-Acetate (PMA; Sigma-Aldrich, St. Louis, MO) at a final concentration of 30 nM for 60 min at 37 $^\circ$C, 5% CO$_2$, or left unstimulated as the control. To detect cellular content of ROS, di-hydro-ethidium (DHE, Sigma-Aldrich, St. Louis, MO; final concentration 1.5 $\mu$M) or di-hydro-rhodamine (DHR; Molecular Probes, Eugene, OR; final concentration 1.5 $\mu$M) were added to the cells. After the incubation, the cell suspensions were washed in cold PBS (4 $^\circ$C), and re-suspended in phosphate buffered saline (PBS, Thermo Fisher, Naerum, Denmark) with 5% FCS. The cells were incubated with anti-CD14-allophycocyanin (APC) (BD Bioscience, Brøndby, Denmark) and near infrared (NIR, Thermo Fisher, Naerum, Denmark) at room temperature, in the dark, for 30 min prior to flow cytometric acquisition.

**Flow cytometry**

From each cell sample, 200,000 events were acquired on a FACS CANTO-II Flow-Cytometer (BD Bioscience, Brøndby, Denmark), and data were analyzed with FlowJo software (version 10). Living monocytes were gated based on their forward-scatter/side-scatter characteristics, CD14-positivity and NIR-negativity. The mean and median fluorescence of DHR (FITC channel, FL-1) and DHE (PE channel, FL-2) intensity were used as quantitative measures of H$_2$O$_2$ and O$_2^-$, respectively.

**Urine samples**

In 32 patients, we also investigated the impact of ruxolitinib therapy on the systemic repair of oxidatively damaged RNA and DNA nucleosides excreted in urine (8-oxo-Guo and 8-oxo-dGuo, respectively). Eleven patients had baseline-samples and all of these had a follow-up ≥6 months. Samples were stored at $-20^\circ$C and analyzed by Ultra Performance Liquid Chromatography (UPLC) with mass spectrometry (MS/MS) as previously published [33]. Analyses were performed at the laboratory of clinical pharmacology, Rigshospitalet/department of clinical pharmacology, Bispebjerg and Frederiksberg Hospital. The outcome variables 8-oxo-Guo and 8-oxo-d-Guo (normalized with creatinine concentration) reflect repaired oxidatively damaged RNA and DNA, respectively [33].

**Statistics**

The statistical analysis was carried out with SAS version 9.4 using SAS Enterprise Guide version 7.1 (SAS Institute Inc., Cary, NC), and $p$ values $<.05$ were considered statistically significant. In order to estimate the effect of ruxolitinib sequentially (repeated measurements), we used the ‘mixed’ model procedure.
Results

The effect of ruxolitinib therapy on circulating cell counts

We first investigated the association between ruxolitinib therapy and concentrations of neutrophils, lymphocytes, and platelets during the course of treatment. We found that changes in peripheral blood counts were independent of time when adjusting for ruxolitinib-dose and that the associations between ruxolitinib-dosing and the relevant blood counts were highly significant (Figure 1). Forty microgram daily intake (DI; standard dosing) resulted in a predicted decrease in neutrophils of \(2.6 \times 10^6\)/L (32% reduction, Figure 1(B)), a predicted reduction in platelets of \(112 \times 10^9\)/L (43% reduction, Figure 1(D)), and a predicted increase in lymphocyte-count of \(0.95 \times 10^6\)/mL (80% increase, Figure 1(F)). Thus, the associations were both statistically significant and clinically relevant.

Figure 1. Neutrophil-, lymphocyte- and platelet counts by time on treatment. Thirty-five patients with myelofibrosis were treated with ruxolitinib followed for up to 36 months, and blood samples were taken sequentially. Shown are the circulating concentrations of (A) neutrophils, (C) lymphocytes, and (E) platelets. (A, C, E) Lines represent cell counts of individual patients with different ruxolitinib dosages shown in different colors (Red: 0–15 mg per day, blue: 20–35 mg per day, black: 40–50 mg per day). (B, D, F) Lines shown are predicted values including 95% upper- and lower confidence limits for cell counts as a function of dosing, calculated using “mixed model” procedures.
Monocyte count did not change significantly during therapy, but showed a tendency towards a dose-associated reduction ($p = .14$, data not shown).

**The effect of ruxolitinib therapy on the content of $O_2^-$, and $H_2O_2$ in circulating monocytes**

PBMCs from the patients with myelofibrosis included in this study were isolated sequentially during the course of ruxolitinib treatment and stained with DHR or DHE (in separate tubes) to detect the content of $H_2O_2$ and $O_2^-$, respectively (Figure 2).

Monocytes displayed a ruxolitinib-dependent decrease in $O_2^-$ content in both non-stimulated ($p < .0001$; Figure 3(A)) and PMA-stimulated samples ($p < .0001$; data now shown). The decrease was time-independent (non-stimulated $p = .23$, PMA-stimulated $p = .29$; data not shown).

The prediction model estimated that the decrease in $O_2^-$ content by monocytes was about 21% at a standard ruxolitinib dosing of 40 mg DI (Figure 3(B)).

We considered the possibility that the reduction in $O_2^-$ content was paralleled by a reduction in the number of circulating monocytes. However, we did not find such an association ($p = .64$ in unstimulated samples). Neither was the reduction in monocyte $O_2^-$ content associated with the levels of neutrophils, the other ROS-producing cell type examined ($p = .62$).

The $H_2O_2$ content in monocytes, non-stimulated ($p = .09$) or stimulated with PMA ($p = .37$), did not change significantly during ruxolitinib, and was not associated with the dosing or time on treatment (data not shown). Furthermore, the intracellular $H_2O_2$ content in monocytes were not associated with monocyte counts ($p = .22$) or neutrophil counts ($p = .39$).

**The effect of ruxolitinib therapy on oxidatively damaged nucleosides**

The urinary excretion of 8-oxo-Guo and 8-oxo-dGuo reflect RNA and DNA damage, respectively. As shown in Figure 4, we did not find any statistically significant changes during treatment with ruxolitinib.

One patient, who showed extremely high values of oxidatively damaged DNA and RNA, transformed into acute myelogenous leukemia (AML) after inclusion in the study but prior to initiation of ruxolitinib (purple, broken lines in Figures 4(A,B)). During the study, she received azacitidine in addition to ruxolitinib, but extremely high values were also found prior to azacitidine therapy. Thus raises the suspicion that the transformation was associated with an increase in oxidatively damaged nucleosides.

The oxidative nucleoside damages on RNA and DNA were not associated with the counts of neutrophils or monocytes nor with the values of ethidium fluorescence reflecting the concentrations of $O_2^-$ or rhodamine fluorescence reflecting concentrations of $H_2O_2$.

**Discussion**

A previously described excess formation of ROS in MF, and association between excess ROS and disease initiation and progression in MF prompted us to undertake this study [13,16]. We investigated the impact of ruxolitinib treatment on ROS formation and ROS-
related consequences in form of oxidatively induced DNA and RNA damage.

We show for the first time that ruxolitinib treatment reduces the spontaneous production of $\text{O}_2^-$ by live monocytes. On standard dosing, 20 mg twice daily, this reduction was about 21%. The PMA-induced stimulation of $\text{O}_2^-$ was also significantly reduced in a dose-dependent, but not time-dependent manner. Thus, both basal and stimulated formation of $\text{O}_2^-$ was impaired by the treatment. On the other hand, ruxolitinib therapy did not decrease intracellular levels of $\text{H}_2\text{O}_2$, neither when assessed as time-on-treatment nor in regard to the dosage given. These findings are interesting, since the formation of $\text{O}_2^-$ is the first step in $\text{H}_2\text{O}_2$ production (Figure 5). Inhibiting subsequent metabolic steps without reducing formation of $\text{O}_2^-$ may create a metabolic bottleneck with shunting of $\text{H}_2\text{O}_2$ through non-enzymatic pathways, e.g. the Haber-Weiss or Fenton reaction. The consequent generation of hydroxyl radicals may have detrimental effects. We have recently reported a highly significant and progressive downregulation of SODs genes using whole blood transcriptional profiling, the most pronounced downregulation being recorded in patients with myelofibrosis [14]. It can be speculated that ruxolitinib may upregulate SODs, thereby promoting a higher turnover rate of $\text{O}_2^-$. Increased $\text{H}_2\text{O}_2$ levels have been shown to reduce treatment response in hepatitis patients receiving IFN [34], and therapeutic reduction of $\text{H}_2\text{O}_2$ might be desirable. Our observations do not support that ruxolitinib has such a potential, however.

Figure 3. Content of $\text{O}_2^-$ in monocytes during ruxolitinib therapy. PMBCs were isolated from 35 patients with myelofibrosis treated with ruxolitinib followed for up to 36 months. Cells were left unstimulated or stimulated with PMA for 60 min, and stained for $\text{O}_2^-$ production using dihydroethidium (DHE) as fluorescent probe. (A) Shown is the median fluorescence intensity (MFI) of DHE in unstimulated, live monocytes in blood samples taken sequentially throughout the study (red lines: 0–15 mg ruxolitinib per day, blue lines: 20–35 mg ruxolitinib per day, black lines: 40–50 mg ruxolitinib per day). (B) Predicted MFI values plotted against ruxolitinib dose using “mixed models” procedures.

Figure 4. Urinary excretion of oxidatively damaged nucleosides during treatment with ruxolitinib. The urinary excretion of 8-oxo-Guo and 8-oxo-dGuo reflecting RNA and DNA damage, respectively, were measured by ultra-performance liquid chromatography and mass spectrometry. Shown is the urinary excretion by the study cohort ($n = 32$) during treatment with ruxolitinib, normalized to creatinine clearance.
We examined the urinary excretion of 8-oxo-Guo and 8-oxo-dGuo. The excretion did not correlate with monocyte concentrations of $O_2^-$ or $H_2O_2$. Importantly, we found no significant change in oxidatively damaged RNA/DNA following treatment with ruxolitinib. However, the urine samples reflect the full-body-scale of oxidatively damaged DNA/RNA, and local effects may not be detected. Other limitations to these measurements are that only a subset of patients had urine samples available for analysis, and MF patients have high comorbidity (including cardiovascular, pulmonary, skeletal and renal diseases), which may affect the systemic load of oxidatively damaged DNA/RNA. The lack of impact on the oxidatively damaged DNA/RNA supports that ruxolitinib is not a disease-modifying agent, even though oxidative modifications of e.g. regulatory RNA cannot be excluded [35].

In this study, we confirm that treatment with ruxolitinib is associated with a reduction in leukocyte and platelet counts, which are elevated in MF [27,36,37]. Often, the drop in platelet counts is a dose-limiting side-effect to the treatment, both in clinical studies and in the common clinical use. Yet, as recently published, even symptomatic patients with platelet counts <50 x 10^9/L may benefit from ruxolitinib treatment [32]. Since elevated leukocyte and platelet counts persist in some patients – albeit being reduced during treatment with ruxolitinib – it can be speculated that the impact of ruxolitinib on elevated leukocyte and platelet counts primarily reflects its anti-inflammatory effects, alleviating inflammation-mediated leukocytosis and/or thrombocytosis, and not greatly influencing the clonally derived leukocytosis and thrombocytosis.

Surprisingly, lymphocyte counts increased with increasing dosages of ruxolitinib. This finding is in contrast to those of most previous studies, in which decreases in particularly the T-cell population have been recorded [38–40]. Since the overall effect of ruxolitinib is anti-inflammatory, the cell types increasing in numbers during ruxolitinib therapy may have anti-inflammatory properties. Indeed, significant negative association was found between the lymphocyte counts and both rhodamine and ethidium. Of note, the numbers of IL-10-producing, regulatory T cells are reduced during therapy with ruxolitinib [38]. Other candidates for lymphocytes with anti-inflammatory properties are regulatory B cells [41].

Of note, the only variable that was associated negatively with both $O_2^-$ and $H_2O_2$ concentrations in monocytes was the lymphocyte count. However, the lymphocyte counts did not associate with DNA or RNA damages.

Our study has several limitations: first, for ethical reasons, it lacks a control group not receiving treatment and all patients included were treated continuously. Patients who discontinued treatment were lost in follow-up – this creates a selection bias where only patients who respond to treatment are included. Second, the main sources of ROS, neutrophils, were not studied, since neutrophils do not survive freezing for subsequent functional studies, which we found...
essential to do in order to minimize inter-assay variation.

In conclusion, we show here for the first time that ruxolitinib treatment of MF patients significantly reduces superoxide radical formation in a dose-dependent manner, but has insignificant impact on the formation of hydrogen peroxide and the level of DNA/RNA damage. Based on these findings, studies of the safety and efficacy of N-acetyl-cysteine (NAC) treatment in combination with IFN and/or ruxolitinib are warranted, based on the ability of NAC to scavenge reactive oxygen species. Indeed, studies of high-dose NAC therapy in chronic inflammatory diseases associated with oxidative stress such as systemic lupus erythematosus, cystic fibrosis, and chronic obstructive pulmonary disorder have given satisfactory responses without harmful side-effects [42–44]. Inclusion of ROS markers in such studies may give important insight into the mechanisms of action of these combination therapies.

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Mads Emil Bjørn performed the cellular studies. Mads Emil Bjørn, Sif Gudbrandsdottir and Christen Lykkegaard Andersen purified PBMCs. Marie Brimnes supervised the flowcytometric analyses. Trine Henrik and Henrik Enghusen Poulsen made the UPLC urinalyses. Hans Carl Hasselbalch and Claus Henrik Nielsen designed the study. Mads Emil Bjørn, Henrik Enghusen Poulsen, Hans Carl Hasselbalch, and Claus Henrik Nielsen analyzed the data and wrote the first draft of the manuscript, which all other authors read critically and approved before submission of the final version.

Potential conflict of interest: Disclosure forms provided by the authors are available with the full text of this article online at https://doi.org/10.1080/10428194.2019.1579323.

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