Evaluation of cytokines and soluble adhesion molecules in patients with newly diagnosed acute myeloid leukemia: the role of TNF-alpha and FLT3-ITD

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Objectives. Acute myeloid leukemia (AML) cells are highly resistant to therapy. The presumed molecular basis of this resistance is the effect of tumor necrosis factor alpha (TNF- α) and other cytokines on endothelial adhesion molecule expression. The aim of this study was to test the hypothesis that cytokines and soluble adhesion molecules correlate in AML.

Methods. Baseline serum levels of 17 cytokines and 5 soluble adhesion molecules were measured in 53 AML patients using biochip array technology. Age, leukocyte count, secondary AML, CRP, FLT3-ITD and remission were variables. Statistical analysis was performed in R version 3.1.2.

Results. VCAM-1 correlated with ICAM-1 ($P < 0.0001$), E-selectin ($P < 0.0001$), leukocyte count ($P = 0.0005$) and TNF- α ($P = 0.0035$). E-selectin correlated with leukocyte count ($P < 0.0001$), P-selectin ($P = 0.0032$) and MCP-1 ($P = 0.0119$). CRP correlated with IL-6 ($P < 0.0001$), leukocyte count negatively correlated with IL-7 ($P = 0.0318$). FLT3-ITD was associated with higher E-selectin ($P = 0.0010$) and lower IL-7 ($P = 0.0252$). Secondary AML patients were older. Failure of induction therapy was associated with significantly higher CRP and lower P-selectin. Leukocyte count ($P < 0.0001$), FLT3-ITD ($P = 0.0017$) and secondary AML ($P = 0.0439$) influenced the principal component.

Conclusions. Leukemic cells can modulate the microenvironment. Cytokine, adhesion molecule levels and leukocyte count correlate in AML. Understanding these mechanisms may form the basis of novel therapeutic approaches.

Key words: cytokines, adhesion molecules, acute myeloid leukemia, TNF-alpha, FLT3-ITD

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INTRODUCTION

The outcomes of AML treatment are beleaguered by the high resistance of malignant clones to therapy. Even high-dose multi-agent chemotherapy and allogeneic stem cell transplantation often fail to prevent relapse. Further, the toxicity of these regimens and the enormous risk of infection during prolonged pancytopenia prevent further dose escalation. Cytokines and adhesion molecules have been studied as markers of immune system activation in many diseases including AML. Experimentally, leukemic cytokines enhance endothelial cell growth in a co-culture of AML and endothelial cells¹. There is evidence that AML activity influences serum levels of cytokines, growth factors and soluble adhesion molecules and promotes angiogenesis^{2,3}. Tumor necrosis factor alpha (TNF- α) and other cytokines are involved in the pathogenesis of acute myeloid leukemia (AML) and high-risk myelodysplastic syndromes (MDS). TNF- α secretion by AML cells and its involvement in the activation of signal transduction pathways and disease progression have been reported^{4,5}. In a prospective study on newly diagnosed AML patients,

lower TNF- α levels were associated with higher rates of complete remission and better event-free survival (EFS) in univariate analyses. However, in multivariate analyses, higher TNF- α levels were not found to be an independent factor predicting clinical outcome but became statistically significant, when leukocyte count was excluded from analyses⁶. AML cells were found to trigger positive feedback loops and activate endothelial cells. Activated endothelial cells express adhesion molecules involved in leukocyte rolling (P- and E-selectin) leukocyte adhesion (VCAM-1, ICAM-1), the same as chemoattractants MCP-1 and IL-8 (ref.^{7,8}). Endothelial cell activation by TNF- α was followed by concomitant induction of VCAM-1, ICAM-1 and E-selectin expression and time-dependent increase in the number of myeloblasts attached to the endothelium⁹. These interactions form the molecular basis of life threatening leukostasis and extramedullary spread of AML. Further, adhesion to activated endothelia may dramatically increase the resistance of AML to chemotherapy and negatively influence treatment outcome¹⁰. Soluble (s) forms of adhesion molecules arise from proteolytic cleavage of surface-expressed molecules¹¹. Increased binding of

sVCAM-1 to $\alpha_4\beta_1$ integrin VLA-4 (Very Late Antigen-4) on myeloblasts was significantly associated with longer overall survival in newly diagnosed adult AML patients¹². In conclusion, AML cell susceptibility to chemotherapy is modulated by dynamic adhesive interactions. To address this issue, we searched for links between pro-inflammatory cytokines, soluble adhesion molecules and other factors important for clinical decisions in newly diagnosed AML patients.

PATIENTS AND METHODS

Patients

A total of 53 newly diagnosed AML patients in the period 2010–2014, 32 with primary AML and 21 with secondary disease, were studied. Further details about patient cohort are summarized in Table 1. The group of secondary AML patients consisted of 17 patients with progression from myelodysplastic syndrome, 2 patients with progression from chronic myelomonocytic leukemia, 1 patient with previous primary myelofibrosis and 1 patient with unclassifiable myeloproliferation. Only patients eligible for induction chemotherapy were included. All patients were tested for cytogenetics and presence of molecular genetic aberrations. Twelve patients were Famosin-like tyrosin kinase 3 – internal tandem duplications (FLT3-ITD) positive. A total of 13 patients underwent leukapheresis due to initial hyperleukocytosis (Leucocyte count $\geq 50 \times 10^9/L$). Patients with acute promyelocytic

leukemia were treated with PETHEMA regimen combining Idarubicin and all-trans retinoic acid (ATRA). All other patients were induced with “3+7” induction chemotherapy consisting of Cytarabine 100 mg/m² per day for 7 consecutive days and Daunorubicin 90mg/m² for the first 3 days of therapy in younger patients. In patients aged ≥ 65 years, Daunorubicin 45-60 mg/m² was administered. Seven patients had initial infection requiring intravenous antibiotic treatment. Complete remission (CR) was evaluated according to NCCN criteria. The study was approved by the local Ethics Committee and performed in accordance with the current Helsinki Declaration. All patients gave their written consent to the study.

Serum collection

Peripheral blood was collected in serum separating tubes, immediately transported to the laboratory and processed within 2 h of collection by centrifugation at 1500rpm x 5 min. All sera were collected before leukapheresis, administration of hydroxyurea or induction therapy. If specimens were not to be analysed immediately, they were stored frozen in small aliquots at below -70 °C as recommended by the Cytokine Array manufacturer. Repeat freeze/thaw cycles were avoided.

METHODS

All analytes were measured by biochip array technology using chemiluminescent sandwich immunoas-

Table 1. Characteristics of patients.

	All patients	Primary AML	Secondary AML
Patients	53	32 (60 %)	21 (40 %)
Age (years)	53.1 $\pm$ 13.4	46.3 $\pm$ 13.3	63.4 $\pm$ 6.4
Sex			
Male	20 (38 %)	14 (44 %)	6 (29 %)
Female	33 (62 %)	18 (56 %)	15 (71 %)
Cytogenetics (risk)			
Favourable	11	11	0
Intermediate	22	11	11
Adverse	20	10	10
White blood cell count ($\times 10^9/L$)	35.6 $\pm$ 39.3	46.8 $\pm$ 50.9	18.4 $\pm$ 19.1
Hyperleukocytosis	13 (25 %)	10 (31 %)	3 (14 %)
FAB type			
M0	3	1	2
M1	11	6	5
M2	25	13	12
M3	4	4	0
M4	6	5	1
M5	3	3	0
M7	1	0	1
Active infection	7 (13 %)	3 (9 %)	4 (19 %)
CRP (mg/L)	47.1 $\pm$ 48.4	39.3 $\pm$ 37.1	59.0 $\pm$ 68.2
FLT3-ITD	12 (21 %)	10 (31 %)	2 (9 %)
No CR after induction	12 (21 %)	4 (13 %)	8 (38 %)

Table 2. Bonferroni-Holm corrected *P* values of Spearman correlations for cytokines & adhesion molecule levels with age, leukocyte count and CRP. IL-8 and IL-10 not shown.

	IL-2	IL-4	IL-6	VEGF	INF-G	TNF-A	IL-1A	IL-1B	MCP-1	EGF	VCAM-1	ICAM-1	E-SEL	P-SEL	IL-3	IL-7	IL-12	IL-13	Age	Leu	CRP
IL-2		1.0000	1.0000	1.0000	1.0000	1.0000	0.1709	0.5601	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
IL-4	1.0000		1.0000	1.0000	1.0000	1.0000	0.0609	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
IL-6	1.0000	1.0000		1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	0.0000
VEGF	1.0000	1.0000	1.0000		1.0000	1.0000	1.0000	1.0000	0.0020	1.0000	1.0000	1.0000	1.0000	0.6044	1.0000	0.3800	1.0000	1.0000	1.0000	1.0000	1.0000
INF-G	1.0000	1.0000	1.0000	1.0000		1.0000	1.0000	1.0000	0.0386	1.0000	1.0000	1.0000	0.9462	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
TNF-A	1.0000	1.0000	1.0000	1.0000	1.0000		1.0000	1.0000	1.0000	1.0000	0.0035	0.0609	0.2495	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	0.5601	1.0000
IL-1A	0.1709	0.0609	1.0000	1.0000	1.0000	1.0000		0.6025	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
IL-1B	0.5601	1.0000	1.0000	1.0000	1.0000	1.0000	0.6025		1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
MCP-1	1.0000	1.0000	1.0000	1.0000	0.0386	1.0000	1.0000	1.0000		1.0000	1.0000	1.0000	0.0119	1.0000	1.0000	0.5102	1.0000	1.0000	1.0000	0.2029	1.0000
EGF	1.0000	1.0000	1.0000	0.0020	1.0000	1.0000	1.0000	1.0000	1.0000		1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
VCAM-1	1.0000	1.0000	1.0000	1.0000	1.0000	0.0035	1.0000	1.0000	1.0000	1.0000		0.0000	0.0000	0.2029	1.0000	1.0000	1.0000	1.0000	1.0000	0.0005	1.0000
ICAM-1	1.0000	1.0000	1.0000	1.0000	1.0000	0.0609	1.0000	1.0000	1.0000	1.0000	0.0000		0.2026	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	0.1011	0.3842
E-SEL	1.0000	1.0000	1.0000	1.0000	0.9462	0.2495	1.0000	1.0000	0.0119	1.0000	0.0000	0.2026		0.0032	1.0000	1.0000	1.0000	1.0000	1.0000	0.0000	1.0000
P-SEL	1.0000	1.0000	1.0000	0.6044	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	0.2029	1.0000	0.0032		1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
IL-3	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000		1.0000	0.0604	0.1719	1.0000	1.0000	1.0000
IL-7	1.0000	1.0000	1.0000	0.3800	1.0000	1.0000	1.0000	0.5102	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000		1.0000	1.0000	1.0000	0.0318	1.0000
IL-12	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	0.0604	1.0000		0.0993	1.0000	1.0000	1.0000	1.0000
IL-13	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	0.1719	1.0000	0.0993		1.0000	1.0000	1.0000	1.0000
Age	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000		1.0000	1.0000	1.0000
Leu	1.0000	1.0000	1.0000	1.0000	1.0000	0.5601	1.0000	1.0000	0.2029	1.0000	0.0005	0.1011	0.0000	1.0000	1.0000	0.0318	1.0000	1.0000	1.0000		1.0000
CRP	1.0000	1.0000	0.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	0.3842	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	

says applied to the Evidence Investigator Analyzer (Randox Laboratories Ltd., Crumlin, UK). The Evidence Investigator Biochip Array technology is used to perform simultaneous quantitative detection of multiple analytes from a single patient sample. The core technology is the Randox Biochip, a solid-state device containing an array of discrete test regions of immobilised antibodies specific to different cytokines and growth factors. A sandwich chemiluminescent immunoassay was employed for the cytokine array. Increased levels of cytokine in a specimen will lead to increased binding of antibody labelled with horseradish peroxidase (HRP) and thus an increase in the chemiluminescent signal emitted. The light signal generated from each of the test regions on the biochip is detected using digital imaging technology and compared to that from a stored calibration curve. The concentration of analyte present in the sample is calculated from the calibration curve.

We evaluated circulating levels of the following 17 cytokines and 5 soluble adhesion molecules: interleukins (IL-1 α , IL-1 β , IL-2, IL-3, IL-4, IL-6, IL-7, IL-8, IL-10, IL-12p70, IL-13, IL-23), vascular endothelial growth factor (VEGF), tumor necrosis factor- α (TNF- α), interferon- γ (IFN- γ), epidermal growth factor (EGF), monocyte chemotactic protein-1 (MCP-1), E-selectin, L-selectin, P-selectin, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). The results are expressed in nanograms per litre (ng/L) for cytokines and micrograms per litre (μ g/L) for adhesion molecules.

Statistical analysis

Statistical analysis was performed in the R statistical programming environment, version 3.1.2 (<http://www.R-project.org>). The levels of L-selectin were at the upper limit of the Array sensitivity in the majority of patients with hyperleukocytosis, similar to several patients with

out hyperleukocytosis. The levels of IL-23 were below the threshold limits for the majority of patients and hence were not included in further analyses. We analysed correlations between levels of cytokines and soluble adhesion molecules and associations with age, leukocyte count, secondary AML, CRP, FLT3-ITD and remission. We also searched for association of these variables and the first principal component (PC1). Spearman correlations were used pair-wise for continuous variables. To test the association between dichotomous and continuous variables the Mann-Whitney U test (ties-adjusted version from R package coin) was used¹³. Fisher's exact test was used for pairs of categorical variables. The Bonferroni-Holm correction¹⁴ was applied where necessary. In all analyses, $P < 0.05$ was considered statistically significant.

RESULTS

Cytokines and soluble adhesion molecules levels

The levels of VCAM-1 strongly correlated with ICAM-1 ($r = 0.67$) and E-selectin ($r = 0.64$) levels (in both cases with corrected $P < 0.0001$). The correlation with P-selectin was moderate and was not significant after Bonferroni-Holm correction. The E-selectin correlated with P-selectin ($r = 0.56$) and ICAM-1 ($r = 0.44$), but only correlation with P-selectin was significant with correction $P = 0.0032$. There were significant correlations between several cytokines and adhesion molecule levels. TNF- α moderately correlated with VCAM-1 ($r = 0.56$, corrected $P = 0.0035$) and ICAM-1 ($r = 0.48$, corrected $P = 0.0609$), correlations with E- or P-selectin were not significant. MCP-1 was moderately negatively correlated with E-selectin ($r = -0.53$) and positively correlated with IFN- γ ($r = 0.50$), corrected $P = 0.0119$ and $P = 0.0386$ respectively. The levels of EGF and VEGF correlated

(corrected $P = 0.002$). The correlation of IL-12 with IL-3 and IL-13 was close to statistical significance ($P = 0.0604$; $P = 0.0993$) after Bonferroni-Holm correction, the same as the correlation of IL-1 α and IL-4 ($P = 0.0609$). The CRP levels strongly correlated with IL-6 levels with $r = 0.67$, corrected $P = 0.0001$. There was no significant correlation of any evaluated analyte with age in this dataset. For all details see Table 2.

Leukocyte count

The leukocyte count correlated strongly with levels of VCAM-1 and E-selectin, which was significant after Bonferroni-Holm correction with $p = 0.0005$ for VCAM-1 and $P < 0.0001$ for E-selectin respectively. The levels of IL-1 β ($P = 0.0048$) and E-selectin ($P = 0.0158$) were increased in hyperleukocytosis. The negative correlations of leukocyte count with IL-7 and MCP-1 were moderate, but for IL-7 this was still significant after Bonferroni-Holm correction ($P = 0.0318$). No other significant correlations between any two continuous variables were found.

FLT3-ITD

The FLT3-ITD mutation was present in 12 cases. The leukocyte count was associated with FLT3-ITD mutation, which remained close to statistical significance after Bonferroni-Holm correction with $P = 0.0614$. In the presence of FLT3-ITD, the E-selectin (corrected $P = 0.0010$, Fig. 1), and to lesser extent P-selectin (corrected $P = 0.0770$, data not shown) were increased, whereas IL-7 was decreased (corrected $P = 0.0252$). IL-1 β was not associated with FLT3-ITD. FLT3-ITD was more frequent in primary AML cases but it was not statistically significant (data not shown). We found no significant association between FLT3-ITD mutation and CR attainment after induction therapy.

Fig. 1. The association of FLT3-ITD mutation and E-selectin, $P = 0.0010$.

Secondary AML and CR attainment

Secondary origin of AML was significantly associated with higher age (corrected $P = 0.0008$). The IL-10 was decreased in secondary AML but not significantly after Bonferroni-Holm correction. Other analytes evaluated in this dataset were not significantly associated with secondary AML origin. The secondary AML cases had less CR rates than primary cases but not significantly after Bonferroni-Holm correction. Failure of induction therapy was associated with higher CRP levels and lower P-selectin levels which were not significant after Bonferroni-Holm correction corrected $P = 0.1032$ and $P = 0.1035$ respectively.

Fig. 2. The association of PC1 with FLT3-ITD ($P = 0.0017$) and secondary AML origin ($P = 0.0439$).

Principal component analysis

The first principal component (PC1) explained 17% of the variation in the cytokine and adhesion molecule levels. The PC1 significantly correlated with leukocyte count ($r = -0.71$, corrected $P = 0.0001$). FLT3-ITD mutation status (corrected $P = 0.0017$, Fig. 2) and secondary AML origin (corrected $P = 0.0439$, Fig. 2) also influenced PC1 significantly. Age, CR attainment and CRP levels had no significant influence on PC1.

DISCUSSION

Altered levels of cytokines and adhesion molecules have been linked to autoimmune diseases, allergies and cancer, including AML (ref.^{15,16}). An inflammatory environment is associated with tumor progression¹⁵ and this shows that tumors are dynamic, interacting systems. Overexpression of cytokines in acute myeloid leukemia patients declines after chemotherapy administration or remission^{2,17}. TNF- α has a pluripotent role in oncogenesis. Supportive care for some cancers, but not AML so far, includes inhibition of TNF signalling through the use of soluble receptors and neutralizing antibodies^{18,19}. In vitro experiments published so far demonstrate that cytokines and adhesion molecules form a unique interacting functional network. As we lacked data on cytokine and adhesion molecule interplay in vivo, we decided to study both systems together in newly diagnosed AML patients. We used serum for this study as recommended by the Array manufacturer. It has been observed that levels of 44 of 80 cytokines, chemokines, and growth factors are significantly different in serum and plasma²⁰.

Our findings are in agreement with the results of studies showing that cytokine and chemokine expression are largely independent of variables, such as age, gender and haemoglobin levels²¹. On the other hand, we found no correlation of the TNF- α levels and leukocyte counts that have been described previously⁶. We also failed to identify any significant changes in cytokine or adhesion molecule levels associated with standard risk stratification of AML. What we consider important is the finding that adhesion molecule levels are influenced as a whole and correlate with leukocyte count and TNF- α levels. TNF- α triggered and p38 MAPK driven endothelial cell activation was demonstrated in a model of leukostasis and inflammatory bowel disease^{7,9}. We found significant positive correlation of TNF- α and VCAM-1, which in vivo, confirmed our expectation of its pivotal role in endothelial cell activation. To the best of our knowledge, this is the first study evaluating the interplay of an inflammatory environment and adhesion molecules in AML patients. On the other hand, leukocyte count significantly correlated with VCAM-1 and E-selectin but not with TNF- α , so there are undoubtedly other factors involved in endothelial cell activation either singly or in combination, e.g. IL-1 β . Both IL-1 β and E-selectin were increased in hyperleukocytosis. IL-1 β was found to stimulate OCI/AML3 cell line proliferation in an autocrine manner²².

We consider the changes associated with FLT3-ITD partially reflect the increased leukocyte count. E-selectin was increased in FLT3-ITD positive cases and correlated with leukocyte count, which was almost significantly higher ($P = 0.0614$) in FLT3-ITD positive cases. So the FLT3-ITD seems to be another factor predicting endothelial cell activation. We suppose the FLT3-ITD cells are highly active and able to modulate the interplay between AML cells, cytokines and endothelial cell overexpression of adhesion molecules. We conclude both TNF- α and the activity of FLT3-ITD positive AML cells are key factors involved in endothelial cell activation. The results of the PC1 analysis support this conclusion. To the best of our knowledge, the interplay between cytokine, adhesion molecules and specific mutation in AML has not been studied so far. At this point, we have to admit that our results are based on analysis of a quite heterogeneous group of 53 AML patients. We consider this study a pivotal trial in this field. To provide more evidence further studies in a larger cohort of patients should be performed.

In this dataset, we failed to identify any cytokine or adhesion molecule associated significantly with CR attainment. On the other hand, we speculate that CRP and P-selectin in predicting CR could be informative in a larger cohort of patients. CRP levels strongly and significantly correlated with IL-6. Some leukemic cells were confirmed to constitutively express IL-6 with possible autocrine or paracrine receptor stimulation²³. Activation of IL-6 signal transduction involves dimerization of IL-6 receptor gp130 subunit, consequently recruitment of gp130-associated protein-tyrosine kinases Jak1, Jak2, and Tyk2 and tyrosine phosphorylation of STAT-3, to a lesser extent STAT-1. Constitutive STAT-3 activation has been demonstrated in AML (ref.²⁴). Thus, in some AML cases CRP levels need not reflect active infection but they also serve as markers of natural disease activity. In one study, the presence of infection correlated with IL-8 levels (nominal $P = 0.01$) but this was not significant after Bonferroni correction²¹. What seems to be the most troublesome is the fact that one can never be sure about infection status of newly diagnosed patients. The presence of infection, either apparent or obscure, is frequent in these patients. In our dataset, especially secondary AML patients had frequent infections at the time of diagnosis. This may limit the use of cytokine and adhesion molecule levels as prognostic markers.

What raises concern is the influence of secondary disease origin, but not of age on PC1. Secondary AML is more frequent in older patients. Nevertheless, due to progress in the treatment of malignancies in younger patients (e.g. Hodgkin's lymphoma, testicular, early breast and prostate cancer) and curative treatment, younger patients also suffer from secondary AML. In a recently published study on adult AML patients, the IL-6 levels inversely and IL-10 levels directly correlated with survival²⁵. CRP levels were not evaluated in that study. We found IL-10 non-significantly decreased in secondary AML. Our results indicate that these findings might be connected.

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Author contributions: TK: literature search; TK, JMH: research design; MV, IK: laboratory procedures; JV, TK: statistical analysis, TK, JMH: manuscript writing; PZ, LJ, JMH: critical revision.

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