Biomarker concentrations in serum and urine of patients with Ocular Tuberculosis – a prospective descriptive study.

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DECLARATION

I declare that this thesis is my own work in design and execution and I have not submitted, in its entirety or in part, in any previous application for a degree or qualification at this or any other university or institute of learning.

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Fredrich J. van der Colff
April 2022

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ABSTRACT

Purpose:

To describe biomarker concentrations in serum and urine of South African patients with ocular tuberculosis (OTB).

Methods:

A prospective study to compare 29 urine and serum biomarkers in 14 OTB patients at a tertiary eye clinic.

Results:

Median age of participants (7 male and 7 female) was 38.5 years (range 25-73). Most biomarker concentrations were significantly higher in serum than in urine ($p < 0.01$). Only two (IL-1RA and IL-2) showed higher concentrations in urine than serum ($p < 0.01$). Three biomarkers (sIL-2Ra, sTNFRI and IFNγ) showed no difference in concentration between urine and serum ($p > 0.05$).

Conclusions:

Most biomarkers tested showed significant differences in concentration between serum and urine and therefore these 2 biofluids cannot be used interchangeably when studying biomarker profiles. One notable exception is IFNγ, as its concentration did not differ between serum and urine.

Keywords: Biomarkers**,** ocular tuberculosis, South Africa, serum, urine

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OPSOMMING

Doelwit:
Om die biomarker konsentrasies in serum en uriene van Suid-Afrikaanse pasiënte met okulêre tuberkulose (OTB) te beskryf.

Metodes:
'n Prospektiewe studie by 'n tersiêre oogkliniek om 29 serum en uriene biomerkers in 14 OTB pasiënte te vergelyk.

Resultate:
Die mediane ouderdom van die pasiënte (7 mans en 7 vrouens) was 38.5 jaar (reikwydte 25 – 73). Meeste biomerker konsentrasies was noemenswaardig hoër in serum as in uriene ($p < 0.01$). Slegs twee (IL-1RA en IL-2) het hoër konsentrasien in die uriene as die serum gewys ($p < 0.01$). Drie biomerkers (sIL-2Ra, sTNFRI and IFNγ) het geen verskil in konsentrasie gewys tussen die uriene en die serum nie ($p > 0.05$).

Gevolgtrekkings:
Meeste biomerkers wat getoets was het noemenswaardige verskille in konsentrasie tussen uriene en serum gewys en kan dus nie afwisselend gebruik word wanneer biomarker profiele bestudeer word nie. Een merkwaardige uitsondering is IFNγ, aangesien sy konsentrasie nie noemenswaardig verskil het tussen uriene en serum nie.

Sleutelwoorde: Biomerkers, okulêre tuberkulose, Suid Afrika, serum, uriene

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1. Introduction

Tuberculosis (TB) infection remains one of the most challenging diseases in Sub-Saharan Africa. South Africa is one of the 30 highest TB burdened countries that together contribute 87% of the estimated TB cases worldwide.[1] According to the World Health Organisation, South Africa alone contributed 3.6% of the global TB cases in 2019[2]. Adding to the burden, South Africa is also among the 14 countries with the highest prevalence of multi-drug resistant TB (MDR-TB) as well as TB/HIV co-infection. Based on results from the first national TB prevalence survey (South African Medical Research Council, 2021), gathered from a nationally representative sample (*n* = 35 191), TB prevalence in 2018 was 737 per 100 000 South Africans. However, given the global impact of the Covid-19 pandemic it is uncertain what the exact prevalence of TB currently is in South Africa. According to the WHO Global Tuberculosis Report 2021, South Africa is one of 16 countries that contributed 93% of the global drop in TB notifications from 2019 to 2020.[3]

Ocular tuberculosis (OTB) is one of the extrapulmonary manifestations of TB and in one study it was found that 6.8% of patients with pulmonary TB also showed signs of ocular involvement.[4] However, a significant proportion of patients present with only ocular involvement without any other TB symptoms and are, therefore, often first diagnosed and treated at eye clinics. The diagnosis of OTB remains a major challenge. The current gold standard for diagnosing TB is direct microscopy and culture of the *Mycobacterium* bacilli. This method is not always practical as cultures may take several weeks to yield positive results. Newer methods include the use of PCR based testing, but they have shown low sensitivity when used on ocular fluids.[5] Alternative methods are, therefore, required to aid in the diagnosis of ocular TB.

Host biomarker analysis has emerged as a promising new tool in the early identification of persons infected with *Mycobacterium tuberculosis* (M.tb). Research on sputum, serum and urine biomarkers in patients with known M.tb infection has shown promising results in diagnosis and treatment monitoring. Isa et al evaluated urine samples alone and found four urine biomarkers that differentiated patients with TB from healthy controls.[7] Previously, Phalane et al compared saliva and serum from the same individuals and found significant differences in saliva biomarkers when compared with serum.[8] A similar study by Jacobs et al identified novel serum and saliva biosignatures in patients with TB.[9] A recent study evaluated biomarkers in the urine of patients with TB, and found a four-marker biosignature (sIL6R, MMP-9, IL-2Ra, IFNγ) with sensitivity of 85.7% and specificity of 94.7%.[10]

At present it is not known to what extent the concentrations of the different biomarkers investigated in the preceding research correspond or differ between the urine and serum of patients diagnosed with OTB. Without this knowledge it is not possible to know whether or not urine concentrations of biomarkers can be used to calculate or extrapolate serum concentrations and vice versa which in turn has implications for the development of point-of-care diagnostic tests in especially resource-limited settings. To the best of our knowledge, this paper is the first to compare urine and serum biomarkers collected from the same patients with OTB.

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2. Materials and Methods

2.1 Study sample and participants

Serum and urine samples were prospectively collected from 21 different patients diagnosed with OTB at a tertiary hospital eye clinic in Cape Town, South Africa. All participants presented with signs of ocular TB and were classified as either having possible, probable or confirmed OTB according to the classification proposed by Gupta et al.[6] Study participants were furthermore included based on the criteria of being 18 years or older that provided consent and enrolled for this study. Patients excluded from this study either declined consent, were younger than 18 years and/or had another cause of uveitis besides TB. Only 14 paired samples of urine and serum were suitable to be included for biomarker analysis.

2.2 Data collection and analysis

Prior to data collection, ethics approval was obtained from the Health Research Ethics Committee at Stellenbosch University (N13/10/146). Urine and serum samples were collected from enrolled patients at the Tygerberg hospital eye clinic from January 2018 to December 2020. All samples were coded, registered on an electronic database, and placed in cryostorage prior to analysis. During June 2021, the urine and serum samples were removed from cryostorage and tested in a controlled environment using the Luminex® platform. The Luminex®XMAP technology platform is a bead-based system allowing for simultaneous analyses of multiple biomarkers in a single specimen. Based on the principles of flow cytometry, the Luminex® platform delivers fast and cost-effective results where up to 100 analytes can be measured in a single microplate well.[11]

For this study, the following 29 biomarkers were tested on both the urine and serum samples: MMP-9, sIL-6Ra, sIL-2Ra, sIL-4R, sIL-6R, sVEGFR3, sCD30, sEGFR, sgp130, sIL-1RI, sIL-1RII, sRAGE, sTNFRI, sTNFRII, sVEGFR1, sVEGFR2, IFNγ, IL-1RA, Il-2, IP-10, MIP-1B, VEGF-A, MDC, Ferritin, A2M, CRP, Fibrinogen, SAP and Haptoglobin.Reagent kits containing all biomarkers were obtained from Merck Millipore (EMD Millipore, Billerica, MA, USA) and were selected based on the potential shown in an earlier study as candidate biomarkers for pulmonary TB.[10]All assays were run on the Bio Plex 200 platform (Bio Rad Laboratories, Hercules, CA, USA). Data on median fluorescent intensities of the beads and subsequent analysis were done using the Bio Plex Manager 6.1 software (Bio Rad Laboratories).

Statistica(TIBCO Software Inc., CA, USA)was used to analyze the data. Mixed model ANOVA was used to compare means between urine and serum samples. Patient numbers were entered as random effect, and sample type(urine/serum) as fixed effect. Normal probability plots were inspected to check normality assumptions, and due to possible outliers, Box-Cox transformations were used to make the data more normally distributed. The mixed model analyses were done using the "lmer" package in R.

A 5% significance level ($p<0.05$) was used as guideline for indicating significant differences.

3. Results

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Corresponding to the national age peak of TB prevalence, the median age of participants (7 males, 7 females) was 38.5 years (range 25 – 73). The majority of patients were diagnosed with possible OTB and only one (participant 4), with confirmed OTB. Only three cases were bilateral. There were five cases each of posterior and panuveitis, and four cases of anterior uveitis. Only four patients, (2 male, 2 female), all 50 years or younger, were HIV positive (Table 1).

Table 1. Study participant demographics and clinical characteristics. (SD = standard deviation)

Participants	Age	Sex	OTB classification	Anatomical distribution	HIV
1	57	M	Possible	Posterior	N
2	30	F	Possible	Posterior	Y
3	73	M	Possible	Panuveitis	N
4	50	F	Confirmed	Panuveitis	Y
5	38	F	Possible	Posterior	N
6	54	M	Possible	Panuvetis	N
7	25	F	Possible	Posterior	N
8	34	F	Possible	Anterior	N
9	32	F	Possible	Panuveitis	N
10	47	M	Possible	Panuveitis	N
11	39	M	Possible	Posterior	Y
12	37	M	Possible	Anterior	Y
13	27	M	Possible	Anterior	N
14	42	F	Possible	Anterior	N
Total (*n* = 14)	Range 27 - 73 Median 38.5 SD ± 13.27	Male (*n* = 7) Female (*n* = 7)	Possible (*n* = 13) Confirmed (*n* = 1)	Anterior (*n* = 4) Posterior (*n* = 5) Panuveitis (*n* = 5)	Yes (*n* = 4) No (*n* = 10)

Out of 29 biomarkers studied only three (sIL-2Ra, sTNFRI and IFNγ) showed no difference in concentration between urine and serum (Figure 1; Table 2).Two other biomarkers (sTNFRII and VEGF-A) tended towards a difference while the remaining 24 biomarkers showed marked differences in concentration ($p<0.01$) (Figure 2; Table 2). Of these, only 2 (IL-1RA and IL-2) showed higher concentrations in urine compared to serum (Figure 3; Table 2).

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Table 2. Biomarker concentrations for urine and serum samples (*n* = 14).

Biomarker	Urine mean ± SD	Serum mean ± SD	*p*-value
Biomarkers more abundantly expressed in serum			
MMP-9	7.288±2.67	12.006±0.67	<0.01
sIL-6Ra	4.421±0.19	4.788±0.15	<0.01
sIL-4R	0.952±0.00	0.966±0.00	<0.01
sIL-6R	4.938±0.33	5.916±0.19	<0.01
sVEGFR3	0.544±0.00	0.547±0.00	<0.01
sCD30	2.748±0.54	3.331±0.44	<0.01
sEGFR	8.758±3.27	18.371±0.69	<0.01
sgp130	4.505±0.15	5.032±0.03	<0.01
sIL-1RI	2.690±1.38	5.211±1.49	<0.01
sIL-1RII	9.417±2.44	14.863±0.78	<0.01
sRAGE	1.938±1.03	3.388±0.56	<0.01
sVEGFR1	2.711±0.08	2.955±0.04	<0.01
sVEGFR2	31.641±16.77	47.547±2.99	<0.01
IP-10	6.703±2.33	9.025± 2.34	<0.01
MIP-1B	2.36±1.98	4.457±1.50	<0.01
MDC	1.495±0.67	2.958±0.23	<0.01
Ferritin	2.426±0.60	3.76±0.28	<0.01
A2M	0.927±0.00	0.928±0.00	<0.01
CRP	2.093±0.52	3.138±0.06	<0.01
Fibrinogen	1.029±0.14	1.433±0.00	<0.01
SAP	0.228±0.00	1.622±0.00	<0.01
Haptoglobin	1.759±0.11	2.116±0.00	<0.01
Biomarkers more abundantly expressed in urine			
IL-1RA	14.06±5.85	2.40±2.62	<0.01
Il-2	0.695 ± 0.25	0.071±0.12	<0.01
No significant difference between biomarkers			
sIL-2Ra	10.202±2.55	10.522±2.41	0.69
sTNFRI	10.654±3.13	10.338±1.25	0.67
IFNγ	1.524±1.69	0.831±2.84	0.45
sTNFRII*	4.523±0.25	4.427±0.13	0.06
VEGF-A*	6.994±1.78	3.955±5.79	0.08

*Biomarkers show a tendency towards higher concentrations in urine.

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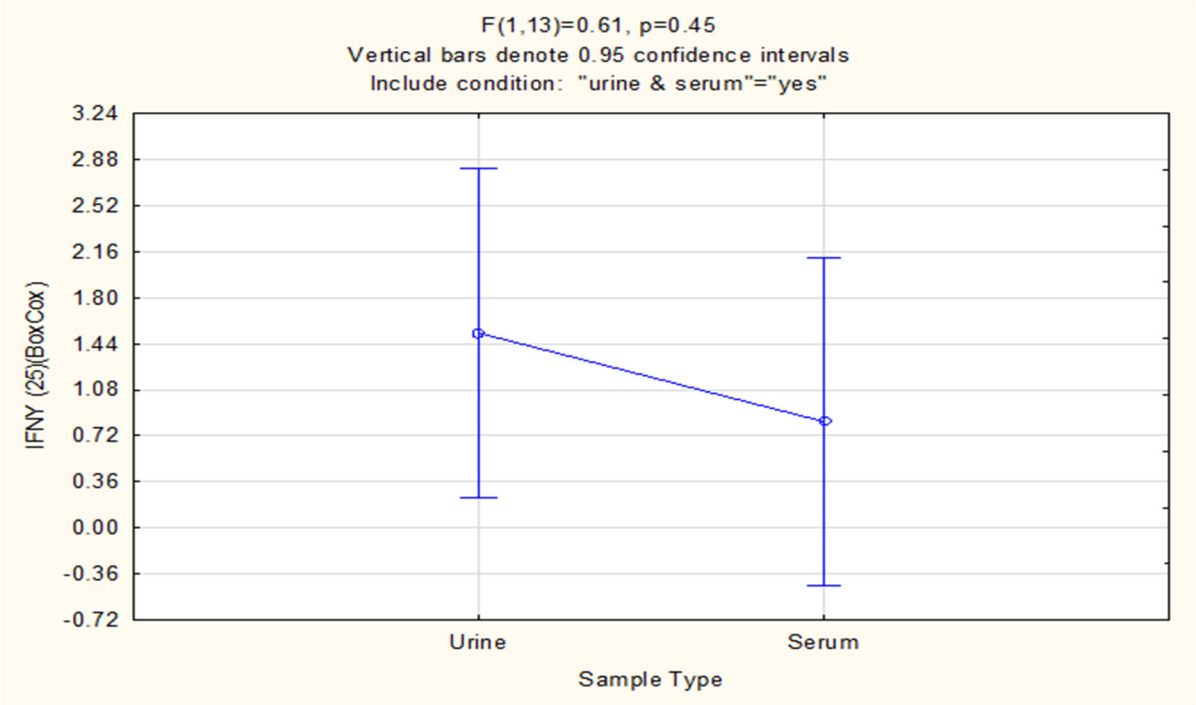

Figure 1. No difference between urine and serum concentrations of IFNγ.

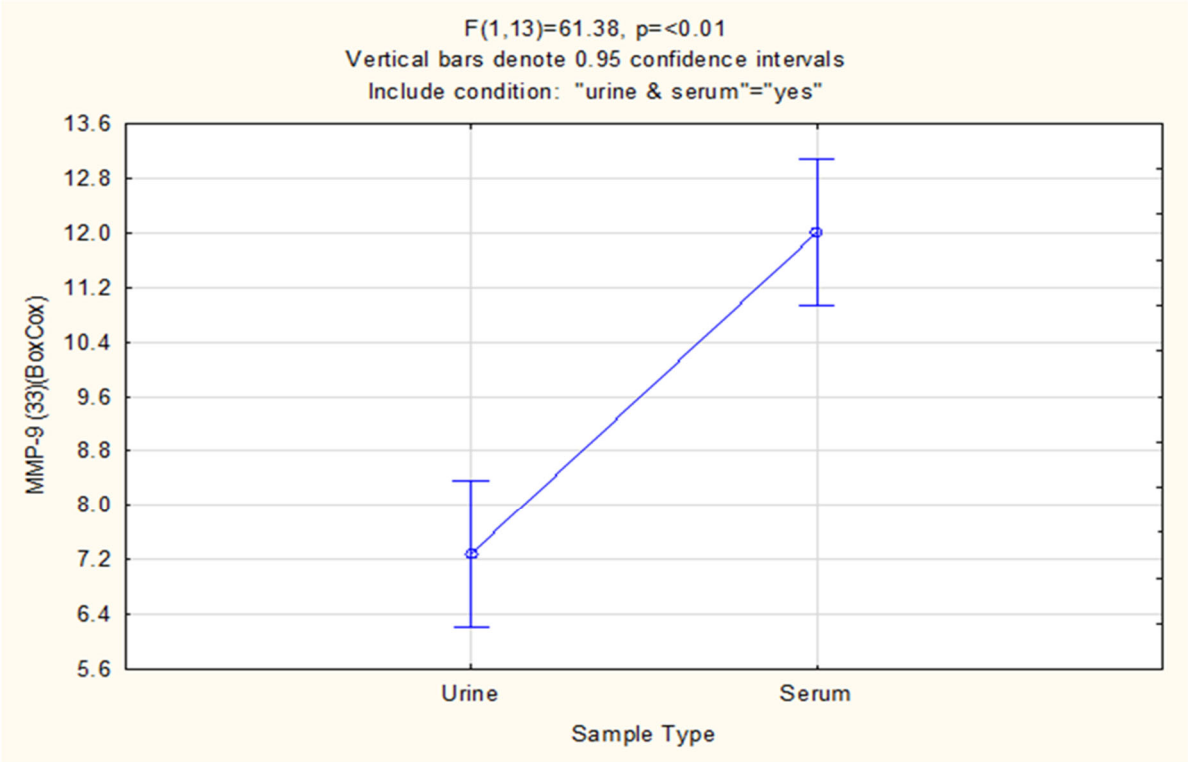

Figure 2. Higher mean serum concentration of MMP-9.

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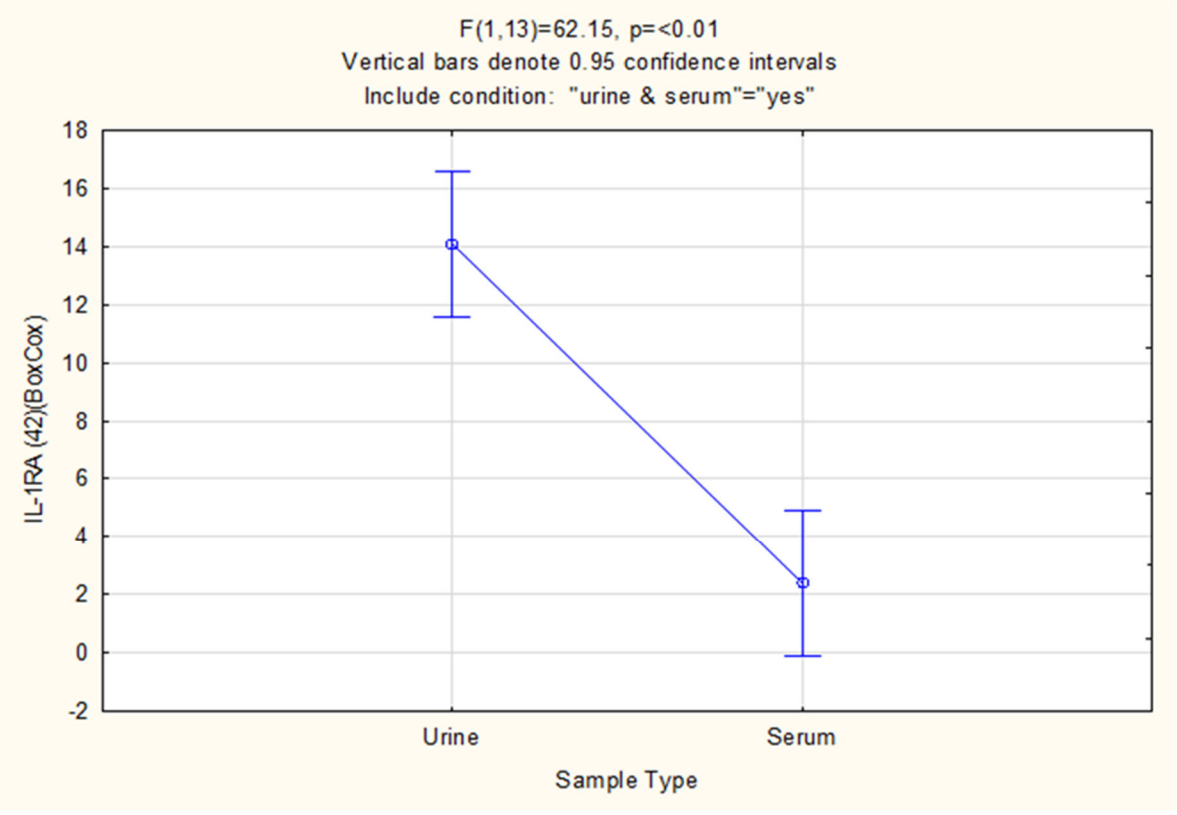

Figure 3. Higher mean urine concentration of IL-1RA.

4. Discussion

Diagnosis of OTB remains challenging and elusive. In many cases, previously undiagnosed TB patients present to eye care centers with ocular involvement, but without any other TB symptoms. The accurate diagnosis of OTB and TB, therefore, is critical for clinicians at eye care centers, especially in high burden TB countries such as South Africa. Detection of TB, and specifically sub-clinical TB, is key to advancing the long-term treatment and management of TB in South Africa and elsewhere.[1] Current methods used to diagnose TB, both direct and indirect, have shown low sensitivity and specificity in detecting OTB.[5] Alternative methods are, therefore, required to help in the diagnosis of OTB.

In this study, urine and serum samples of OTB patients were compared using 29 biomarkers. The concentrations of most biomarkers studied differed markedly in urine and in serum with the majority being much lower in the urine. The findings from this study support those of a previous TB study[10] at the same facility, also reporting significant differences in biomarker levels of serum and saliva. In this study, only two biomarkers, IL-1RA and Il-2, showed significantly higher concentrations in urine than in serum. Three biomarkers showed no significant difference in the serum and urine concentrations, while two biomarkers tended towards a difference.

In most instances, it is, therefore, not possible to extrapolate urine concentrations from serum values and vice versa and most different biofluids therefore are not interchangeable when

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developing OTB biosignatures. However, of pertinent interest is the IFNγ biomarker, currently used in IFNγ release assays (IGRA) to diagnose TB, that was equally expressed in urine and serum (p = 0.45). IFNγ was also part of a four-marker biosignature along with sIL-6R, MMP-9 and IL-2Ra that diagnosed TB with area under curve (AUC) of 0.96, sensitivity of 85.7% and specificity of 94.7% in HIV-positive patients.[10] It is therefore not inconceivable that going forward it could be possible to develop non-invasive, cost-effective and time-efficient urine tests to detect OTB – especially for use in resource-poor countries with high TB prevalence.

To validate these findings, more research is, however, required to investigate the reliability of the urine biomarkers. Due to time constraints and difficulties to collect data during the Covid-19 pandemic, the sample size of this study is small and it is suggested to use a larger sample of OTB patients in future studies. Nonetheless, this study contributes to advancing the understanding of biomarkers for OTB.

5. Conclusion and recommendations

The majority of biomarkers tested showed significant differences in concentration between serum and urine and therefore these 2 biofluids cannot be used interchangeably when studying biomarker profiles. One notable exception is IFNγ as its concentration did not differ between serum and urine. It is recommended that more research with larger sample sizes be done to validate and elaborate on the findings of this study.

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