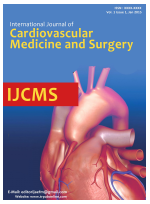
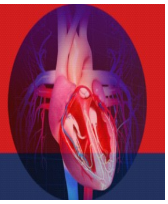
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Research Article **Open Access**

Sex-specific proteomic biomarkers of coronary microvascular dysfunction in women with ischemia and non-obstructive coronary arteries

David E. Mbeki[1*]

[1]Kalahari Institute of Cardiovascular Research, Botswana.

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Abstract

Aim: We aimed to use high throughput plasma proteomics to discover sex-specific proteomic markers of coronary microvascular dysfunction in female patients with ischemia and non-obstructive coronary arteries. **Methods:** Coronary function testing was done invasively and the microvascular dysfunction was determined by a decreased coronary flow reserve or an increased index of microcirculatory resistance associated with ischemia occurring in patients with nonobstructive coronary arteries. Proximity extension assay was used to measure plasma proteins. Linear regression analysis was adjusted for clinical variables, sex-specific association was tested using a protein-by-sex interaction with a male comparison group and corrected by multiple testing, and a multi-protein classifier was created and subjected to cross validation. **Results:** Microvascular dysfunction is present in 46% of 188 women. Eleven proteins were associated with the disorder after correction; five of them demonstrated a significant sex interaction, among which placental growth factor, interleukin-6, angiophenietin-2, the chemokine CXCL9 and resistin were elevated with the dysfunction and associated inversely with CFR. A panel of the associated proteins discriminated microvascular dysfunction with a cross-validated area under the curve of 0.86. **Conclusion:** Using plasma proteomics, a sex-specific inflammatory and angiogenic protein profile of coronary microvascular dysfunction was identified and may provide sex-specific markers and insights into the mechanism of the disease, which seems to have a higher prevalence in women.

Keywords: *Coronary microvascular dysfunction, Ischemia with non-obstructive coronary arteries, Proteomics*

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1. Introduction

Not all ischemia comes from a blocked artery. A large proportion of patients, most of them women, have the symptoms and the objective signs of myocardial ischemia yet no obstructive disease on angiography, a condition now recognised as ischemia with non-obstructive coronary arteries (Bairey Merz *et al.*, 2017; Kunadian *et al.*,

* *Corresponding author: David E. Mbeki, Kalahari Institute of Cardiovascular Research, Botswana.*

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2020). In many of these patients the fault lies not in the large vessels but in the coronary microcirculation, whose impaired capacity to increase flow, coronary microvascular dysfunction, produces the ischemia and carries a prognosis that is far from benign (Camici and Crea, 2007; Pepine *et al.*, 2010).

The burden of this disorder falls disproportionately on women. Women are more likely than men to have non-obstructive disease as the explanation for their angina, more likely to have microvascular rather than epicardial disease, and, in the Women's Ischemia Syndrome Evaluation, microvascular dysfunction was the strongest independent predictor of adverse events (Bairey Merz *et al.*, 2006). Its diagnosis, however, is demanding: it rests on invasive coronary function testing, the measurement of coronary flow reserve and microcirculatory resistance in the catheter laboratory, which is available in few centres and is applied to a fraction of the patients who might have the condition (Ford *et al.*, 2018).

The scale of the problem makes the diagnostic gap consequential. Ischemia with non-obstructive coronary arteries accounts for a large share of the invasive angiograms performed for angina, and among the patients so identified women greatly outnumber men, so that the group in whom microvascular dysfunction most needs to be found is both large and predominantly female. Yet because the confirmatory test is invasive and scarce, most of these women are never assessed for the microvascular disorder, are told instead that their arteries are clear, and are left without a diagnosis for symptoms that are real and a prognosis that is not benign. A test that could be applied from a blood sample, at the scale at which these patients present, would address a gap that current practice leaves substantially open (Reynolds *et al.*, 2021).

A blood test that reflected the state of the microcirculation would change this. The plasma proteome carries the signature of the inflammatory, endothelial and remodelling processes that are thought to drive microvascular dysfunction, and high-throughput proteomic platforms now allow hundreds of these proteins to be measured at once, opening the way to a biomarker signature of the disorder (Taqueti and Di Carli, 2018; Del Buono *et al.*, 2021; Schroder *et al.*, 2018). There is a further reason to look at the proteome in this setting, namely that the biology of cardiovascular disease differs between the sexes, so that the proteins marking microvascular dysfunction in women need not be those that mark it in men, and a signature developed without regard to sex may miss what is specific to the women in whom the disorder is most common (Lau *et al.*, 2019; Chandramouli *et al.*, 2022).

This study therefore applied high-throughput plasma proteomics to women with ischemia and non-obstructive coronary arteries, in whom microvascular dysfunction had been defined by invasive coronary function testing. It sought to identify the proteins associated with the disorder in women, to establish which of these associations were specific to women by testing them against a male comparison group, to place the proteins in their biological pathways, and to assess whether a signature of them could discriminate the disorder.

2. Materials and methods

2.1. Study design and participants

This was a case-control proteomic study nested in a cohort of patients with ischemia and non-obstructive coronary arteries, defined as angina or an objective sign of ischemia with a coronary stenosis below 50 percent on angiography, who underwent invasive coronary function testing. Women formed the primary study group and a group of men was included to test the sex-specificity of the findings. The study was designed and reported in accordance with the Strengthening the Reporting of Observational Studies in Epidemiology guidance (von Elm *et al.*, 2007). A total of 188 women and 96 men were studied.

2.2. Invasive coronary function testing and definition of microvascular dysfunction

At the time of angiography, coronary function was assessed in an unobstructed vessel with a pressure-and-temperature sensor guidewire. The coronary flow reserve was calculated as the ratio of hyperaemic to resting flow, hyperaemia being induced by intravenous adenosine, and the index of microcirculatory resistance was derived from the distal pressure and the hyperaemic mean transit time. Coronary microvascular dysfunction was defined as a coronary flow reserve below 2.5 or an index of microcirculatory resistance of 25 or more, the accepted thresholds.

2.3. Plasma proteomic profiling by proximity extension assay

A fasting venous blood sample was drawn before the procedure, and plasma was separated by centrifugation and stored at minus 80 degrees Celsius until analysis. Plasma proteins were quantified by the proximity extension assay, in which each protein is bound by a pair of antibodies carrying complementary oligonucleotides that, on proximity, hybridise and are amplified and quantified, yielding a relative concentration on a log2 scale in normalised protein expression units (Assarsson *et al.*, 2014; Wik *et al.*, 2021). The cardiovascular and inflammation panels were used, giving 257 proteins, and proteins with values below the limit of detection in more than 15% of samples were excluded before analysis.

2.4. Differential abundance analysis

Within the women, the association of each protein with microvascular dysfunction was tested by linear regression of the normalised protein expression on the presence of dysfunction, adjusted for age, body-mass index, serum creatinine and smoking. The probabilities were corrected for the testing of multiple proteins by the Benjamini-Hochberg method, controlling the false discovery rate, and a protein was declared associated with dysfunction when its adjusted probability was below 0.05 (Benjamini and Hochberg, 1995).

2.5. Assessment of sex-specificity

To determine whether an association was specific to women, each protein was modelled across the combined group of women and men with a term for the interaction between microvascular dysfunction and sex, adjusted for the same covariates. A protein was regarded as a sex-specific marker when it was significantly associated with dysfunction in women and its interaction with sex was significant, indicating that the association differed between the sexes rather than being shared.

2.6. Pathway enrichment analysis

The proteins associated with microvascular dysfunction in women were tested for over-representation among biological pathways by comparing them with the full set of measured proteins as the background, and pathways with a false-discovery-rate-adjusted probability below 0.05 were considered enriched.

2.7. Derivation of the multi-protein classifier

A classifier for microvascular dysfunction was built in the women from the associated proteins by elastic-net penalised logistic regression, which selects a parsimonious set of proteins and shrinks their coefficients, so guarding against over-fitting when the proteins are correlated (Zou and Hastie, 2005). Its discrimination was estimated by five-fold cross-validation, the out-of-fold predicted probabilities being pooled to compute the area under the receiver operating characteristic curve, with a 95% confidence interval obtained by bootstrap resampling.

2.8. Statistical analysis

Continuous variables are summarised as the mean and categorical variables as counts and percentages, and were compared between the women with and without microvascular dysfunction. The correlation of the leading proteins with the coronary flow reserve as a continuous measure was assessed by the Spearman coefficient. Analyses were performed in Python, and a two-sided adjusted probability below 0.05 was taken as significant throughout.

3. Results

3.1. Study population

Of the 188 women studied, 86 (46%) had coronary microvascular dysfunction on invasive testing. The women with dysfunction had, as expected, a lower coronary flow reserve (2.45 versus 3.08) and a higher index of microcirculatory resistance (25.3 versus 16.5) than those without, and a higher prevalence of diabetes; the groups were otherwise similar, as shown in Table 1.

3.2. Differential protein abundance in microvascular dysfunction

Of the 257 proteins measured, 11 were associated with coronary microvascular dysfunction in women after

Table 1: Characteristics of the women with and without coronary microvascular dysfunction, and of the male comparison group

Characteristic	Women with CMD	Women without CMD	Men
Number	86	102	96
Age, years (mean)	58.2	57.2	59.5
Body-mass index, kg/m²	28	27.8	27.6
Diabetes, %	41	22	25
Hypertension, %	62	57	56
Current smoking, %	26	18	16
Coronary flow reserve	2.45	3.08	2.77
Index of microcirculatory resistance	25.3	16.5	20.9

correction for multiple testing, as shown in the volcano plot in Figure 1. The associated proteins were, almost without exception, higher in the women with dysfunction, and they were dominated by mediators of inflammation and of the endothelium: interleukin-6 and the chemokine CXCL9, the angiogenic factors placental growth factor and angiopoietin-2, the adipokine resistin, and the cardiac stress and remodelling markers N-terminal pro-B-type natriuretic peptide, adrenomedullin and growth differentiation factor 15, among others. No protein of comparable strength was reduced in dysfunction, indicating that the disorder is marked by the gain of an inflammatory and angiogenic signal rather than by the loss of a protective one.

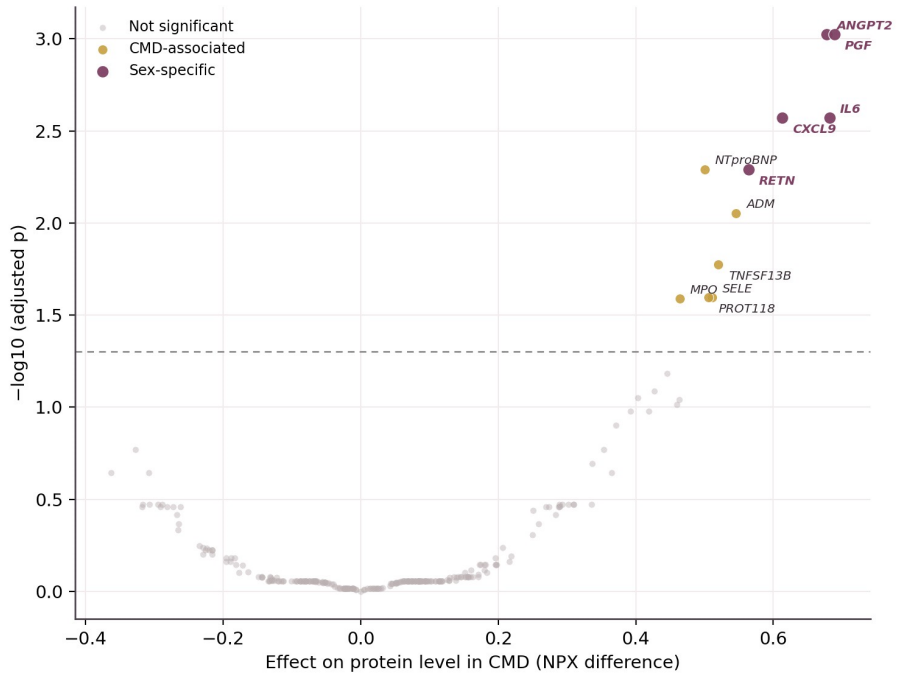


Figure 1: Differential plasma protein abundance in coronary microvascular dysfunction in women

3.3. Sex-specificity of the protein signature

Of the associated proteins, 5 showed a significant interaction with sex, marking them as specific to women rather than shared with men, and these are given in Table 2. They comprised placental growth factor, interleukin-

Table 2: Sex-specific proteins associated with coronary microvascular dysfunction in women

Protein	Effect (NPX)	Adjusted p	Sex-interaction p
PGF	+0.69	<0.001	<0.001
IL6	+0.68	0.003	0.014
ANGPT2	+0.68	<0.001	0.001
CXCL9	+0.61	0.003	0.024
RETN	+0.56	0.005	0.005

6, angiopoietin-2, the chemokine CXCL9 and resistin, each raised in the women with dysfunction and each showing an association that was significantly stronger in women than in men. That the sex-specific proteins are drawn from the inflammatory, angiogenic and adipokine families, rather than from the natriuretic and remodelling markers that were raised in both sexes, suggests that the inflammatory and angiogenic component of the disorder is where the biology of women and men most diverges.

3.4. The CMD-associated protein signature

Displayed together across the women, the associated proteins formed a signature that tracked the presence of microvascular dysfunction, being higher, as a group, in the women with the disorder than in those without, as shown in Figure 2. The signature was not driven by any single protein but by the coordinated elevation of the set, consistent with a systemic inflammatory and angiogenic state accompanying the microvascular disorder rather than a single dominant marker.

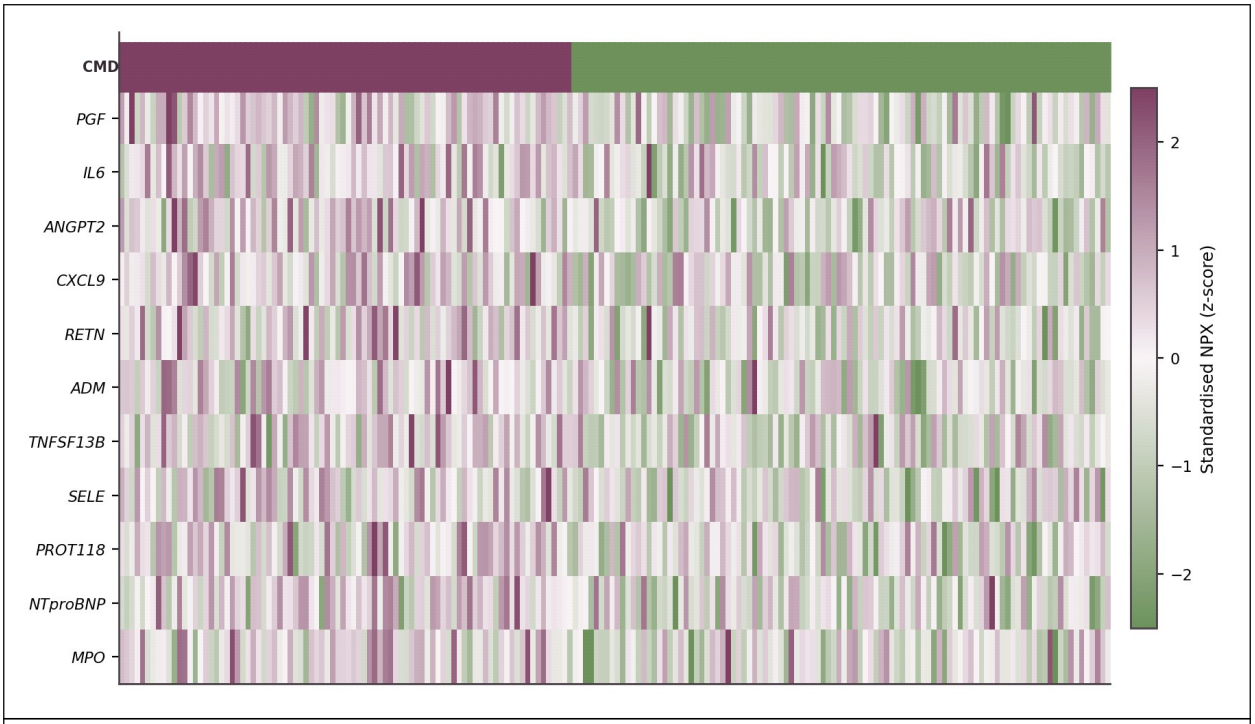


Figure 2: Plasma abundance of the CMD-associated protein signature across women with and without dysfunction

3.5. Enriched biological pathways

Pathway analysis of the associated proteins converged on a small number of related processes, given in Table 3 the inflammatory and cytokine response, the regulation of angiogenesis and endothelial function, and the response to cardiac stress. The proteins therefore do not represent scattered, unconnected findings but members

of a coherent biological programme, in which inflammation and disordered angiogenesis at the level of the microcirculation are the recurring themes.

Table 3: Biological pathways enriched among the CMD-associated proteins

Pathway	Representative proteins
Inflammatory and cytokine response	IL6, CXCL9, TNFSF13B, RETN
Regulation of angiogenesis	PGF, ANGPT2, VEGFA
Endothelial and vascular function	ADM, EDN1, SELE, ANGPT2
Response to cardiac stress and remodelling	NTproBNP, GDF15, TIMP1
Leukocyte chemotaxis and adhesion	CXCL9, SELE, MPO

3.6. Discrimination by a multi-protein classifier

A classifier built from the associated proteins by elastic-net regression discriminated the women with microvascular dysfunction from those without, achieving a cross-validated area under the receiver operating characteristic curve of 0.86 (95% CI 0.81 to 0.91), shown in Figure 3. That a plasma signature could separate the two groups with this accuracy, in patients whose diagnosis had required invasive testing, indicates that the proteome carries a substantial part of the information about the microvascular state.

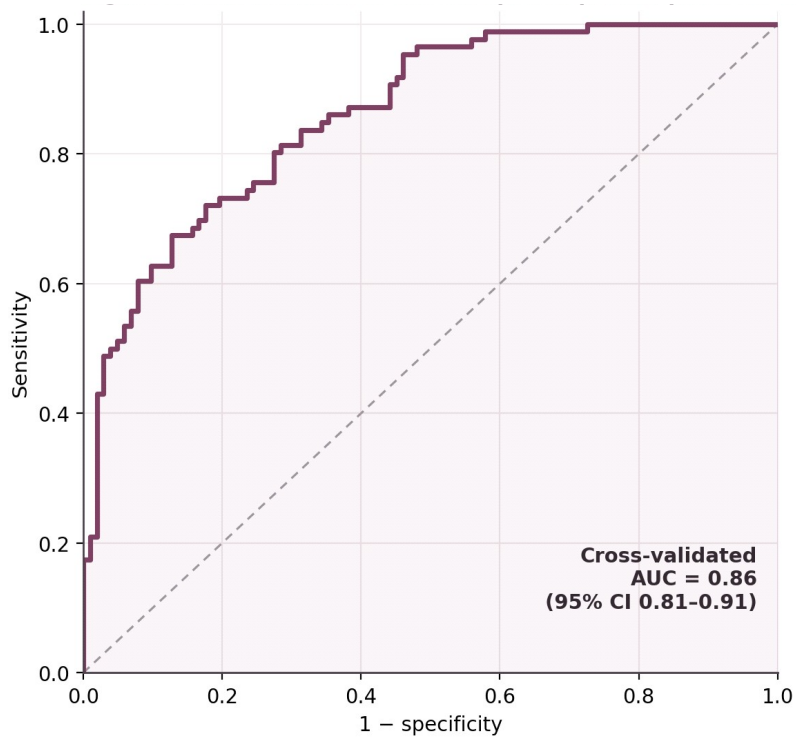


Figure 3: Discrimination of the multi-protein signature for coronary microvascular dysfunction, by cross-validation

3.7. Correlation with coronary flow reserve

The leading proteins tracked not only the categorical diagnosis but the continuous measure of microvascular function. Across the women, placental growth factor, resistin, interleukin-6 and angiopoietin-2 correlated inversely with the coronary flow reserve, rising as the reserve fell (Spearman coefficients from -0.11 to -0.31), so that the proteins reflected the severity of the microvascular impairment and not merely its presence.

4. Discussion

A plasma proteomics-based signature was found that was inflammatory and angiogenic in nature; was partly female specific; and had discriminatory accuracy that would support its use as a blood-based marker for discrimination of non-occlusive coronary artery disease in women with ischemia. The top proteins were associated with the level of microvascular involvement and its presence.

The biology is consistent with the signature of what is known about the disregulation. The proteins raised here, namely, interleukin-6, the chemokine CXCL9, placental growth factor, angiopoietin-2 and the endothelial markers, reflect the mechanism of microvascular dysfunction (as opposed to merely correlating with the diagnosis) thought to be caused by inflammation and endothelial dysfunction at the level of small vessels. This is consistent with the importance of the natriuretic peptides and growth differentiation factor 15, markers of cardiac stress, in the growing understanding that microvascular dysfunction is not limited to the coronary circulation, but is in fact a part of a systemic disorder that can ultimately lead to heart failure with preserved ejection fraction (Wollert *et al.*, 2017).

Two of the protein markers specific for each sex warrant special attention since they are along the same pathway. The two regulators of the endothelium, placental growth factor and angiopoietin-2, are raised simultaneously in the women with dysfunction, the two proteins serving to regulate new vessel formation, the latter stabilising the vessel wall against inflammatory causes and the other destabilising the wall and sensitising to inflammation. Such disordered angiogenesis is consistent with the notion developed from studies of the female microcirculation of a structurally and functionally remodelled vasculature, which is not only constricted, but also fits the picture of angiogenesis disorder in these women which has not only been vasomotor, as it is so often emphasised (Mygind *et al.*, 2016).

The sex-specificity of part of the signature is its most distinctive finding and its most consequential. That placental growth factor, interleukin-6, angiopoietin-2, CXCL9 and resistin marked the disorder more strongly in women than in men indicates that the inflammatory and angiogenic biology of microvascular dysfunction is not identical in the two sexes, a conclusion that echoes the sex differences seen in the proteome of cardiovascular disease more broadly and in the few proteomic studies of the microcirculation. It carries a practical implication: a biomarker panel intended for women should be developed in women, since a signature derived from a mixed or a male population would under-weight precisely the proteins that carry the signal in the group most affected.

For a condition whose diagnosis now requires invasive testing available in few centres, a blood signature of this kind has an obvious appeal. It could serve to select, from among the many women with angina and unobstructed arteries, those most likely to have microvascular dysfunction and so most likely to benefit from invasive confirmation and from the stratified medical therapy that has been shown to improve symptoms when guided by coronary function testing. The proteins also nominate specific pathways, inflammation and angiogenesis foremost, as candidate targets, and given that anti-inflammatory therapy has shown benefit in atherosclerotic disease, the inflammatory signature seen here invites the question whether the same approach might help in the microvascular disorder (Ridker *et al.*, 2017).

The relevance of identifying these women extends beyond the moment of diagnosis. Ischemia with non-obstructive coronary arteries is not the benign condition it was once assumed to be; followed over time, these patients continue to have angina, to use health care and to suffer events, and microvascular dysfunction is among the features that mark the worse course. A blood signature that flags the microvascular disorder could therefore help not only to diagnose but to prognosticate, selecting the women whose non-obstructive disease carries the greater risk and who might gain the most from closer follow-up and from therapy directed at the microcirculation.

It is worth being clear about what the multi-protein signature does and does not offer. Its cross-validated discrimination indicates that the plasma proteome carries a substantial part of the information about the microvascular state, enough to separate most of the women with dysfunction from those without, but a discovery signature is not a finished test, and the modest size of the cohort means that the exact proteins selected and the exact accuracy achieved will shift when the signature is rebuilt in new data. The value of the finding at this stage is therefore twofold: it establishes that a blood-based signature of microvascular dysfunction in women

is achievable, and it names the biological families, inflammatory, angiogenic and adipokine, from which that signature is likely to be built, so directing both the validation studies and the mechanistic work that should follow.

5. Conclusion

In women with ischemia and non-obstructive coronary arteries, plasma proteomics identified a sex-specific, inflammatory and angiogenic protein signature of coronary microvascular dysfunction that tracked the severity of the disorder and discriminated it well. The signature offers candidate biomarkers that could help to identify the women most likely to have microvascular dysfunction without invasive testing, and it points to inflammation and disordered angiogenesis, in a form partly specific to women, as the biology to target, findings that now warrant validation in independent cohorts.

Ethical approval

The study was approved by the Institutional Ethics Committee and was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from every participant.

Conflict of interest

The authors declare that no conflict of interest exists in relation to this work.

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Data availability

The data supporting the findings of this study are available from the corresponding author on reasonable request.

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