Tampon-Based Menstrual Fluid Sampling Enables Non-Invasive Detection of Neurological, Hormonal, and Inflammatory Biomarkers

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OBJECTIVE

This study aims to establish menstrual fluid as a novel, non-invasive biospecimen for the measurement of clinically relevant systemic and reproductive biomarkers, including those reflective of neuroinflammatory and hormonal states. Recognizing the inherent variability in blood contribution to menstrual effluence, we specifically address the unmet need for robust normalization strategies in this matrix. We assessed transferrin as a quantitative marker of blood content in menstrual effluence and to enable normalization of biomarker measurements. We examined whether normalization restores expected biological relationships and enables reliable detection of neurological biomarkers, including neurofilament light chain [1,2]. In parallel, we analyzed NEFL gene expression across menstrual samples spanning the inflammatory resolution phase of menstruation.

BACKGROUND

Menstrual effluence is a biologically rich but analytically complex specimen that contains variable contributions from circulating blood, endometrial tissue, immune infiltrates, and cervicovaginal secretions [3]. Because this material can be collected noninvasively during routine menstruation, it offers a potential window into both reproductive and systemic biology. However, interpretation of soluble biomarkers in menstrual effluence is complicated by substantial differences in blood fraction between samples. Visual inspection alone reveals that menstrual samples range from lightly colored fluid containing little visible blood to dark samples dominated by blood. This variability introduces dilution effects that can obscure biological relationships between analytes and physiological variables.

A strategy to correct for blood fraction variability would enable reliable interpretation of biomarkers in this matrix. Transferrin is an iron transport protein derived from circulating blood that is largely absent from nonvascular secretions. For this reason, transferrin has the potential to function as an internal indicator of blood content and to provide a normalization factor for biomarker measurements.

Once analytical variability is addressed, interpretation of molecular signals within menstrual effluence also requires a framework that reflects the biological dynamics of menstruation. During the menstrual phase the endometrium transitions from inflammatory tissue breakdown toward coordinated tissue repair. The Inflammatory Resolution Score (IRS) is a transcriptomic axis that orders menstrual samples along this trajectory from inflammatory breakdown to repair [4]. Positioning samples along this biological continuum allows gene expression signals to be interpreted in the context of menstrual physiology rather than as static measurements.

Establishing reliable normalization in menstrual effluence is particularly important for neurological biomarkers. Neurofilament light chain is a well validated marker of neuroaxonal injury that is measurable in blood and cerebrospinal fluid [5]. Whether neurological biomarkers can be detected and interpreted in menstrual effluence has not previously been established.

METHODS

Menstrual effluence samples were collected from consented participants using a standardized tampon-based collection kit with whole-cell preservation buffer, designed for at-home use [6]. Tampons were worn for approximately four hours, then sealed and returned by mail for processing. Supernatants from N = 99 samples (91 participants) were analyzed by automated chemiluminescence immunoassay on the Siemens Healthineers Atellica® Analyzer for NfL, IL-6, estradiol (eE2), anti-Müllerian hormone (AMH), and an immunoturbidimetric chemistry assay for transferrin (Trf)*. NfL was quantified in technical triplicate where sample volume permitted; replicate means were used as the primary analyte value. Fluid color grade (C2 through C5, light to very dark) was used as an independent visual proxy for blood content.

Transferrin-normalized AMH was computed as the ratio AMH/Trf. NEFL gene expression was analyzed in an independent RNA-seq dataset: a cross-tissue comparison of menstrual blood versus venous whole blood (N = 102; 57 menstrual, 45 venous), [2]. IRS was computed for all menstrual RNA-seq samples as previously described [2]. Associations were assessed by Spearman rank correlation. Group comparisons used Mann-Whitney U and Kruskal-Wallis tests. Partial Spearman correlations were computed using rank-based residuals, which is the appropriate approach for controlling confounders in rank-correlation analyses.

*Disclaimer: The assays and features mentioned herein are not commercially available in all countries and/or for all modalities. Their future availability cannot be guaranteed.

RESULTS

Menstrual effluence samples displayed substantial variability in visible blood content when classified using the standardized five level color grading scale (C1–C5), with samples ranging from lightly colored fluid containing little visible blood to dark samples dominated by blood (Figure 1). This variability highlights a central analytical challenge in interpreting soluble biomarkers in menstrual effluence.

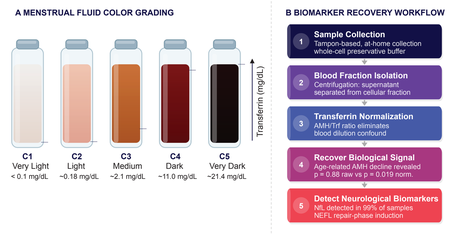

Figure 1. Standardized visual grading of menstrual effluent blood content. Menstrual effluent samples were assigned a standardized color grade from C1 to C5 reflecting increasing visible blood fraction. This variability illustrates the need for normalization when interpreting soluble biomarkers in menstrual samples.

To determine whether transferrin could serve as a quantitative indicator of blood fraction, transferrin concentrations were compared across samples and color grades. Transferrin concentration increased monotonically with increasing color grade, with a Spearman correlation of 0.63 and a significance level of 8.1×10^{-12}, and a Kruskal Wallis significance level of 4.9×10^{-9} (Figure 2). Median transferrin concentrations ranged from 0.18 mg/dL in lighter samples to 21.4 mg/dL in darker samples. These results confirm that transferrin provides a quantitative measure of blood fraction in menstrual effluence.

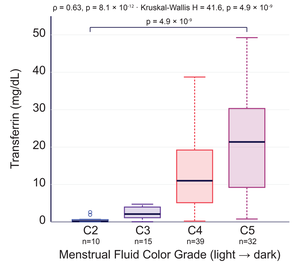

Figure 2. Transferrin concentration increases monotonically with menstrual fluid color grade, validating it as a quantitative blood content index. Boxplots show transferrin (mg/dL) across color grade bins C2 through C5 (N = 96 samples; C2 n = 10, C3 n = 15, C4 n = 39, C5 n = 32). Color grade is an independent visual assessment of sample darkness used as a proxy for blood content. Horizontal lines denote medians. Individual data points are overlaid. Spearman ρ = 0.63, p = 8.1×10^{-12}; Kruskal-Wallis p = 4.9×10^{-9}. Significance bracket indicates Mann-Whitney p < 0.001 for C2 vs. C5.

Inflammatory analytes behaved in a manner consistent with blood derived signal. IL-6 concentrations showed a strong positive correlation with transferrin with a Spearman correlation of 0.73 and a significance level of 1.7×10^{-17} (Figure 3a). In contrast, neurofilament light chain showed a weaker but significant association with transferrin with a Spearman correlation of 0.41 and a significance level less than 0.001 (Figure 3b). This pattern indicates that blood fraction contributes substantially to inflammatory signal in menstrual effluence, while NfL variability cannot be explained solely by differences in blood content.

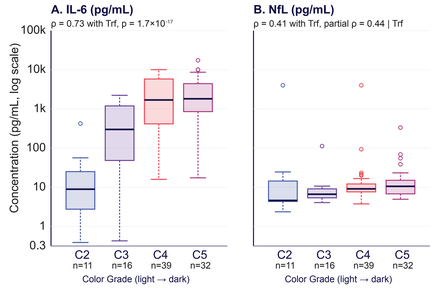

Figure 3. IL-6 tracks blood content strongly; NfL does not, indicating a non-blood-derived component. Side-by-side boxplots show IL-6 (left panel) and NfL (right panel) concentrations on a $\log_{10}$ scale across color grades C2 through C5 (N = 96 for both analytes). IL-6 correlates strongly with transferrin (ρ = 0.73, p = 1.7×10^{-17}), consistent with blood-driven inflammatory signal. NfL shows a weaker transferrin correlation (ρ = 0.41, p < 0.001), and maintains detectable levels across all color grades, suggesting a source beyond peripheral blood admixture.

Having established transferrin as an indicator of blood fraction, we next asked whether normalization could recover biological relationships that would otherwise be obscured by matrix variability. Raw anti-Müllerian hormone concentrations showed no association with age with a Spearman correlation of –0.03 and a significance level of 0.88. After normalization to transferrin, AMH divided by transferrin was significantly lower in individuals aged thirty-five years or older compared with those younger than thirty-five years, with a significance level of 0.019 in a cohort of thirty-two individuals (Figure 4). This finding recapitulates the well-established decline in ovarian reserve with age and demonstrates that transferrin normalization can restore interpretable biological signal [6].

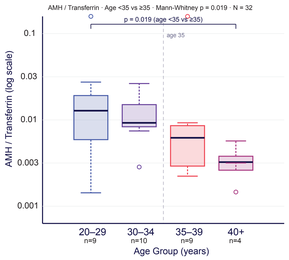

Figure 4. Transferrin normalization unmasks the age-dependent AMH decline that is invisible in raw concentrations. Boxplots show AMH normalized to transferrin (AMH/Trf, ng/mL per mg/dL Trf) on a $\log_{10}$ scale across four age bins: 20 to 29, 30 to 34, 35 to 39, and 40 and older (N = 32; bin sizes 9, 10, 9, 4). A dashed vertical line marks age 35, the threshold for the primary group comparison. Raw AMH showed no age relationship (ρ = –0.03, p = 0.88). Normalized AMH/Trf was significantly lower in participants aged 35 and older (Mann-Whitney p = 0.019). Medians decline stepwise: 0.013, 0.009, 0.006, 0.003 across bins.

Within this normalized analytical framework, neurological biomarkers were readily detectable in menstrual effluence. Neurofilament light chain protein was detected in ninety eight of ninety-nine samples, corresponding to a detection rate of ninety nine percent, with concentrations ranging from 2.4 to 4,000 pg/mL and a median concentration of 8.9 pg/mL. Across the full physiological range of estradiol concentrations, including samples with near zero hormone levels, NfL correlated with estradiol with a Spearman correlation of 0.31 and a significance level of 0.017 in fifty-nine samples. The association remained significant after controlling for transferrin with a partial correlation of 0.32 and a significance level of 0.014 (Figure 5).

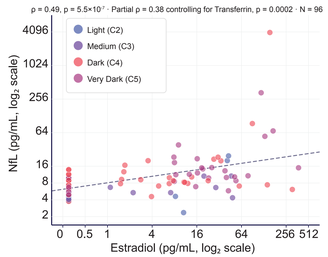

Figure 5. NfL correlates with estradiol in menstrual effluent, and this association persists after controlling for blood content. Scatter plot of NfL versus estradiol on a $\log_2$ scale for the 59 samples with detectable estradiol (eE2 > 0 pg/mL). Points are colored by fluid color grade (C2 through C5). Dashed line shows the linear regression fit. Spearman ρ = 0.31, p = 0.017; partial Spearman ρ = 0.32, p = 0.014, controlling for transferrin via rank-based residuals. Thirty-eight samples with estradiol below the lower limit of detection (< 0.25 pg/mL) are excluded from this figure; their exclusion reflects the low-estrogenic hormonal state of menstruation.

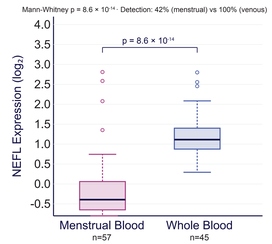

Figure 6. NEFL transcript levels are specifically suppressed in menstrual blood compared to venous whole blood. Boxplots show NEFL gene expression ($\log_2$ normalized counts) for menstrual blood (N = 57) and venous whole blood (N = 45). Individual data points are overlaid. Medians: 0.41 (menstrual) vs. 1.91 (venous). Mann-Whitney p = 8.6×10^{-14}. Detection rate (expression > 0.5 $\log_2$ counts) was 42% in menstrual samples versus 100% in venous samples, arguing against a dilution explanation and suggesting active transcriptional suppression in the uterine compartment.

Tointerpret transcriptional dynamics within menstrual effluence, we positioned RNA sequencing samples along the Inflammatory Resolution Score, a transcriptomic trajectory that reflects the transition from inflammatory tissue breakdown toward coordinated endometrial repair[2]. Across tissues, NEFL transcript levels were significantly lower in menstrual blood than in venous blood with a significance level of 8.6×10^{-14} (Figure 6). Within menstrual effluence samples, NEFL expression increased modestly but significantly along the inflammatory resolution trajectory with a Spearman correlation of 0.7 and a significance level of 0.0001 across fifty-seven samples (Figure 7). Samples positioned in the high-resolution state showed 12.93-fold higher NEFL expression compared with samples in the low-resolution state with a significance level of 3.8×10^{-6}.

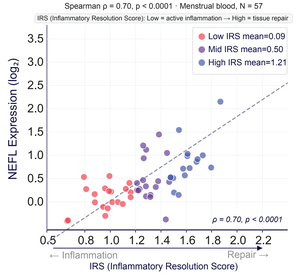

Figure 7. NEFL expression is induced during the tissue repair phase of menstruation, coupling its upregulation to inflammatory resolution. Strip plot showing NEFL expression ($\log_{10}$ scale) across three IRS tertile bins (Low, Mid, High) from the RNA-seq longitudinal menstrual cohort (N = 57 samples). Mean expression bars are overlaid. Mean expression: Low 0.0937, Mid 0.4975, High 1.2122 (12.93-fold enrichment in High vs. Low). Mann-Whitney p = 3.8×10^{-6} (High vs. Low); Spearman ρ = 0.70, p < 0.0001 across the continuous IRS axis.

CONCLUSIONS

Menstrual effluence contains substantial variability in blood fraction that complicates interpretation of soluble biomarkers. Transferrin functions as an indicator of blood content within this matrix and can support normalization to correct for dilution effects.

Using normalization, established biological relationships such as the age-related decline in anti-Müllerian hormone can be recovered from menstrual samples. Within this normalized analytical framework, neurofilament light chain is consistently detectable in menstrual effluence and shows variability that is not fully explained by blood fraction alone.

Transcriptomic analysis further demonstrates that NEFL expression varies across the inflammatory resolution trajectory of menstruation as defined by the Inflammatory Resolution Score. Together these findings establish an analytical and biological framework for interpreting biomarkers in menstrual effluence and highlight the potential of menstrual sampling as a noninvasive approach for studying endocrine, inflammatory, and neurological biology.

References

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