

Evaluation of the Circulating Angiome in Cancer: Translational Assessment of a 44-Plex Angiogenesis Biomarker Panel

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Introduction

Angiogenesis is a hallmark of tumor progression. Dysregulated angiogenesis in the tumor microenvironment leads to leaky, poorly perfused vessels, hypoxia, immune evasion, and therapeutic resistance. Despite widespread use of anti-angiogenic agents, no predictive biomarker identifies likely responders. Prior biomarker efforts focused on limited vascular factors, missing the multifaceted nature of angiogenic signaling. MSD developed a 44-plex panel to enable high-throughput profiling of pro- and anti-angiogenic biomarkers addressing key stages of angiogenesis (Figure 1).

- Hypoxic Insult:** Tumor hypoxia stabilizes HIF signaling, inducing secretion of pro-angiogenic growth factors (e.g., VEGF-A, VEGF-C, PlGF, HGF, Ang-2) that initiate vascular remodeling and endothelial activation.
- Basement Membrane Degradation:** Proteolytic remodeling of the extracellular matrix enables endothelial cell invasion, mediated by enzymes such as MMP-2, MMP-9 (active) and matrix-associated proteins including Osteopontin and Pentraxin-3.
- Tip Cell Migration:** Chemokine-driven guidance of endothelial tip cells directs sprouting toward angiogenic signals, with key mediators including SDF-1 α (CXCL12), MCP-1 (CCL2), Fractalkine (CX3CL1), MIG (CXCL9), and IP-10 (CXCL10).
- Tube Formation:** Endothelial proliferation and organization into nascent vascular structures are regulated by growth factor signaling through pathways involving FGF2, PDGF-BB, Ang-1, VEGFR-2 (KDR), and Tie-2.
- Regulation of Vessel Size:** Vessel maturation, stabilization, and permeability are controlled by endothelial adhesion molecules and signaling regulators such as VEGFR-1 (FLT1), ICAM-1, VCAM-1, E-selectin, P-selectin, vWF, and TGF- β isoforms.
- Tumor Vasculature:** Sustained angiogenesis within the tumor microenvironment is driven by inflammatory and immune-modulatory signals, including TNF- α , IL-6, IL-1 β , GM-CSF, G-CSF, M-CSF, RANTES (CCL5), Galectin-9, S100A12, and CRP, supporting aberrant vascular growth and remodeling.

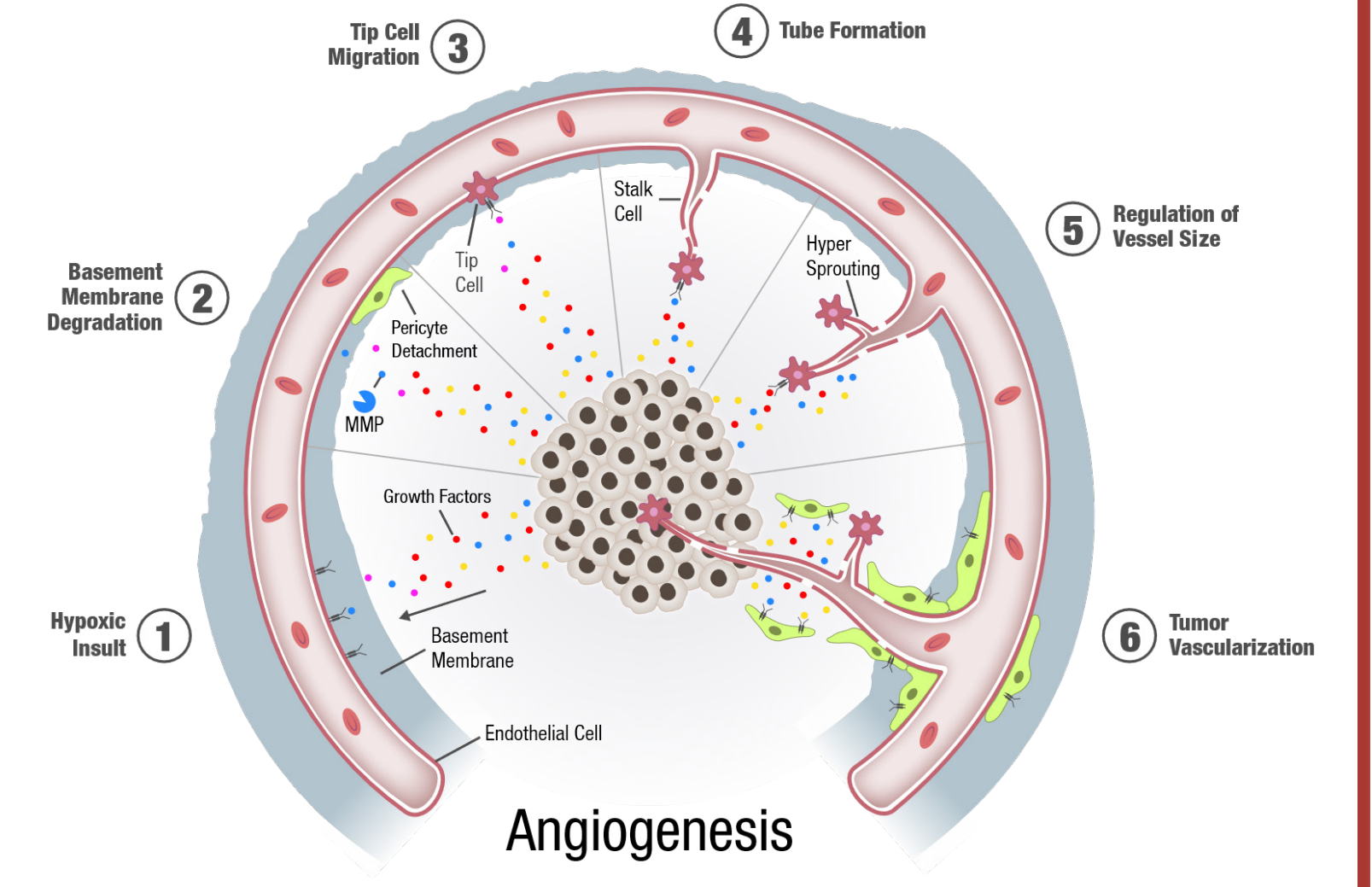


Figure 1. Schematic representation of key stages of angiogenesis.

Methods and Approach

Multiplex Assay Platform and Detection Technology
A 44-plex panel of circulating angiogenesis biomarkers ("Angiome panel") was assessed using the MESO SCALE DISCOVERY[®] (MSD) electrochemiluminescence (ECL) platform. Assays were configured on U-PLEX[®] plates, where linker-based capture chemistry enables flexible assembly of multiplex panels and grouping of analytes based on optimal sample dilution requirements (Figure 2 and Table 1).

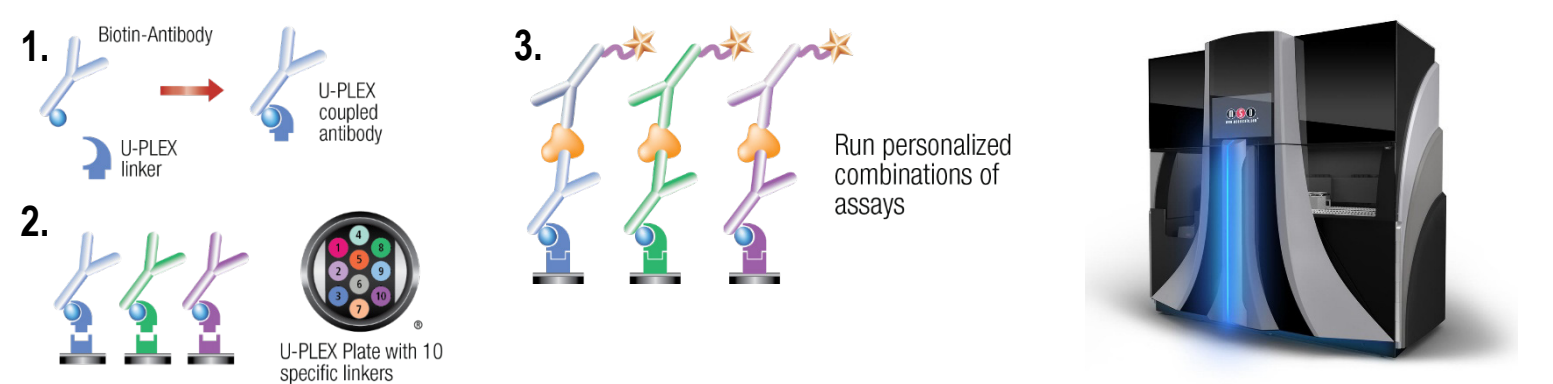


Figure 2. U-PLEX Technology: coupling, coating and assay workflow on the MESO Parsec R 5000. Biotinylated capture antibodies are conjugated to unique U-PLATE linkers (Step 1), enabling their directed immobilization onto prefabricated spots within each well of a U-PLATE plate (Step 2). Multiple linker-antibody conjugates are combined to generate a customized multiplex panel. Following sample incubation, analytes are captured and detected using SULFO-TAG[®]-labeled detection antibodies (Step 3), producing electrochemiluminescent signals proportional to analyte concentration. All assay steps, from plate coupling through signal detection and plate readout, were performed on the MESO Parsec R 5000. This flexible configuration allows simultaneous, quantitative measurement of multiple targets from a single sample.

All measurements were performed using <100 μ L of plasma per sample, enabling efficient multiplex profiling across a broad dynamic range. TGF- β isoforms were quantified following a pre-analytical acid dissociation step to release latent complexes. All samples were assayed according to assay protocols as shown in Figure 2.

Assays were executed on the MESO[®] Parsec R 5000MM analyzer, ensuring standardized, high-throughput processing and reproducible assay performance. ECL signal detection provides high sensitivity and quantitative resolution across low- and high-abundance analytes.

Analytical Characterization and Validation

- Analytical performance of the Angiome panel was evaluated using both recombinant protein controls and endogenous plasma samples to ensure accurate quantification across diverse analytes and concentration ranges.
- Precision:** Intra- and inter-assay variability was assessed using quality controls and pooled plasma samples.
 - Accuracy and Recovery:** Spike and recovery experiments were performed on assays requiring less than 100-fold dilution using recombinant-protein spiked diluent and pooled human plasma.
 - Dilution Linearity:** Evaluated using four plasma samples at four dilutions.
 - Sensitivity and Dynamic Range:** Lower and upper limits of detection (LLOD, TOC) were established relative to observed biological concentrations.

Sample Sources and Study Design

- Plasma samples were provided by Duke University from healthy volunteers and oncology cohort. Samples supported analytical validation, biological stability assessment, and translational pharmacodynamic analysis:
- Healthy volunteer (longitudinal) cohort:** Longitudinal plasma samples from 28 individuals (N=224 samples) were obtained from a previously characterized study (Liu, Cancer Epidemiol Biomarkers Prev, 2025). Data were analyzed using a mixed linear model to partition intra- and inter-individual variance. Intraclass correlation coefficients (ICC) were calculated using both unadjusted and covariate-adjusted models (age, sex, BMI, fasting status).
 - Oncology cohorts:**
 - CaboMab study (NCT0047330):** KRAS wt metastatic colorectal cancer treated with cabozantinib and panitumumab; baseline (n=27) and C2D1 (n=19).
 - XAG study (NCT02008383):** metastatic esophagogastric cancer treated with oxaliplatin, capecitabine, and bevacizumab; baseline (n=49) and C4D1 (n=26).

Matched baseline and on-treatment plasma samples from oncology cohorts (CaboMab and XAG) were analyzed to assess therapy-induced modulation of circulating angiogenesis biomarkers.

For each analyte, changes from baseline were calculated within subjects and normalized using z-score transformation to enable cross-analyte comparison across differing concentration ranges.

Pharmacodynamic responses were visualized using heatmaps, where rows represent individual biomarkers and columns represent subjects. Color scaling reflects relative upregulation or downregulation from baseline, enabling identification of coordinated, pathway-level responses within and across cohorts.

Analytical Characterization, Performance and Validation

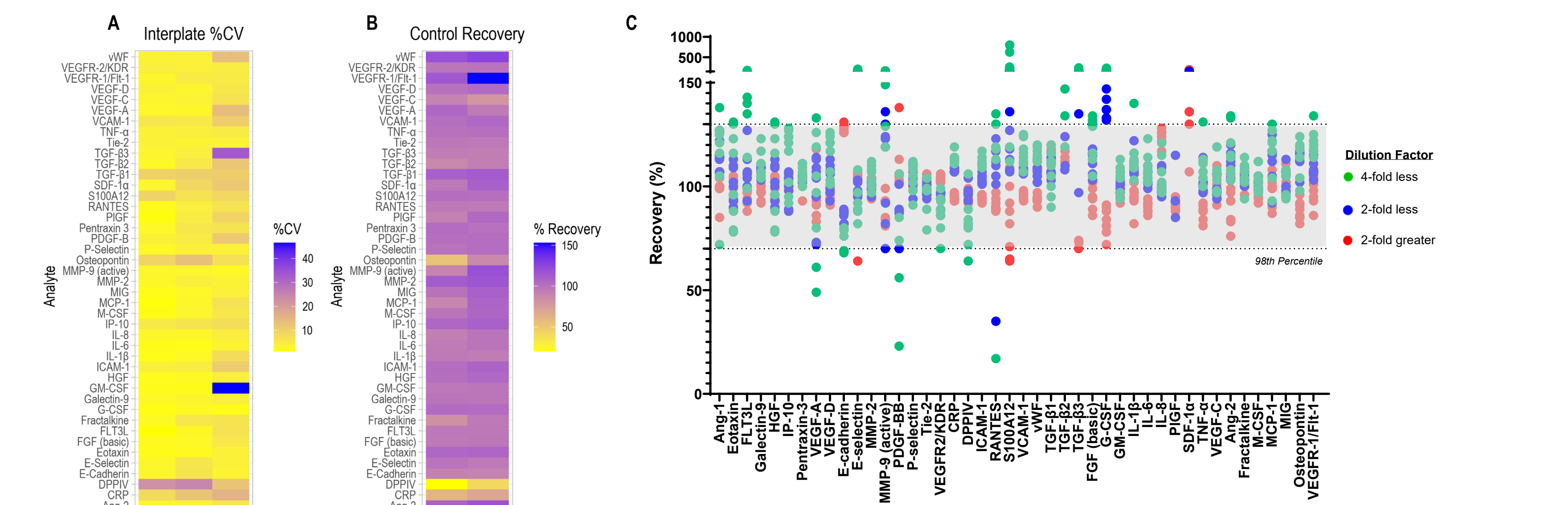


Figure 3. Analytical performance of the 44-plex panel demonstrates robust precision, accuracy, and reproducibility across multiplexed assays. (A) Heatmap of inter-plate %CV for duplicate measurements across QC samples (QC1-QC3) over 11 assay runs, summarizing assay precision across analytes. (B) Heatmap of control recovery (% recovery) for recombinant protein controls (CC01-CC02), illustrating accuracy and consistency of quantification across the panel. (C) Dilution linearity assessed across multiple plasma samples using assay-specific dilution factors, demonstrating proportional signal response across concentration ranges across the 98th percentile (represented by the shaded area). Across analytes, low inter-plate variability and consistent recovery profiles indicate reliable multiplex performance, while dilution linearity confirms quantitative accuracy across a broad dynamic range.

- High reproducibility (%CV <5% for 95% of analytes, recovery between 80-120% for 90% of analytes) across the Angiome enables confident comparison of biomarkers across samples and studies.
- When allowing ≥ 3 dilution levels to meet recovery criteria ($\geq 75\%$ samples within 70-140%), $\sim 90\%$ of assays demonstrated acceptable linearity, indicating robust performance across the usable dynamic range.
- Quantitative accuracy is maintained across diverse biomarker classes, supporting reliable measurement of both low- and high-abundance proteins.
- Consistent signal scaling across concentrations confirms that observed differences reflect true biological variation rather than assay artifacts.
- Robust performance in plasma matrices enables direct application to translational studies without loss of analytical fidelity.

Robust Angiome 44-plex Panel Performance Across Healthy and Cancer Plasma

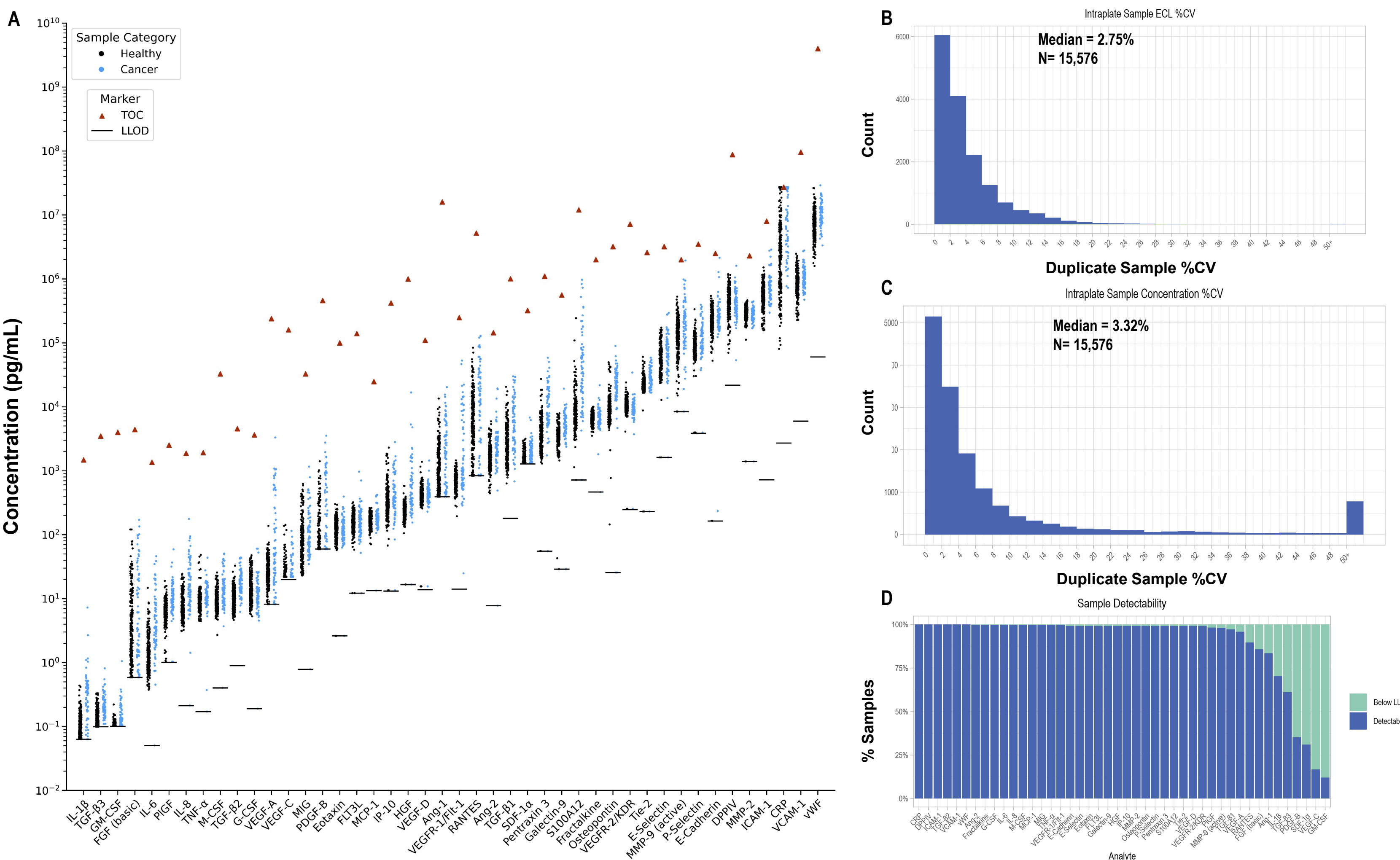


Figure 4. Analytical performance of the Angiome 44-plex panel across healthy and cancer plasma samples. (A) Concentration distributions for all analytes measured in duplicate across samples, with lower limits of detection (LLOD) and calibrator ranges (top of curve, TOC) indicated, demonstrating quantification across ~ 8 -9 orders of magnitude. (B-C) Intra-plate precision assessed from duplicate measurements, showing light reproducibility with median %CVs of 2.7% (ECL signal) and 3.3% (calculated concentration). (D) Percent detectability across samples, with a median of $\sim 80\%$ across all samples.

Precision: Duplicate measurements demonstrate exceptional reproducibility across all analytes, with median %CVs of 2.7% (ECL) and 3.3% (concentration). This level of precision enables clear separation of biological signal from technical noise, supporting confident detection of subtle changes.

Dynamic Range: Using multiple sample dilutions and grouping assays by expected analyte abundance, the 44-plex workflow supports quantification across biomarkers spanning ~ 8 -9 orders of magnitude in concentration.

Detectability: The panel achieves greater than 90% median detectability across healthy and cancer samples, ensuring consistent, biologically relevant coverage across heterogeneous sample populations and minimizing missing data that can confound downstream analyses.

Defining Stable and Dynamic Angiogenesis Biomarkers Using Interclass Coefficient Analysis and Longitudinal Profiling

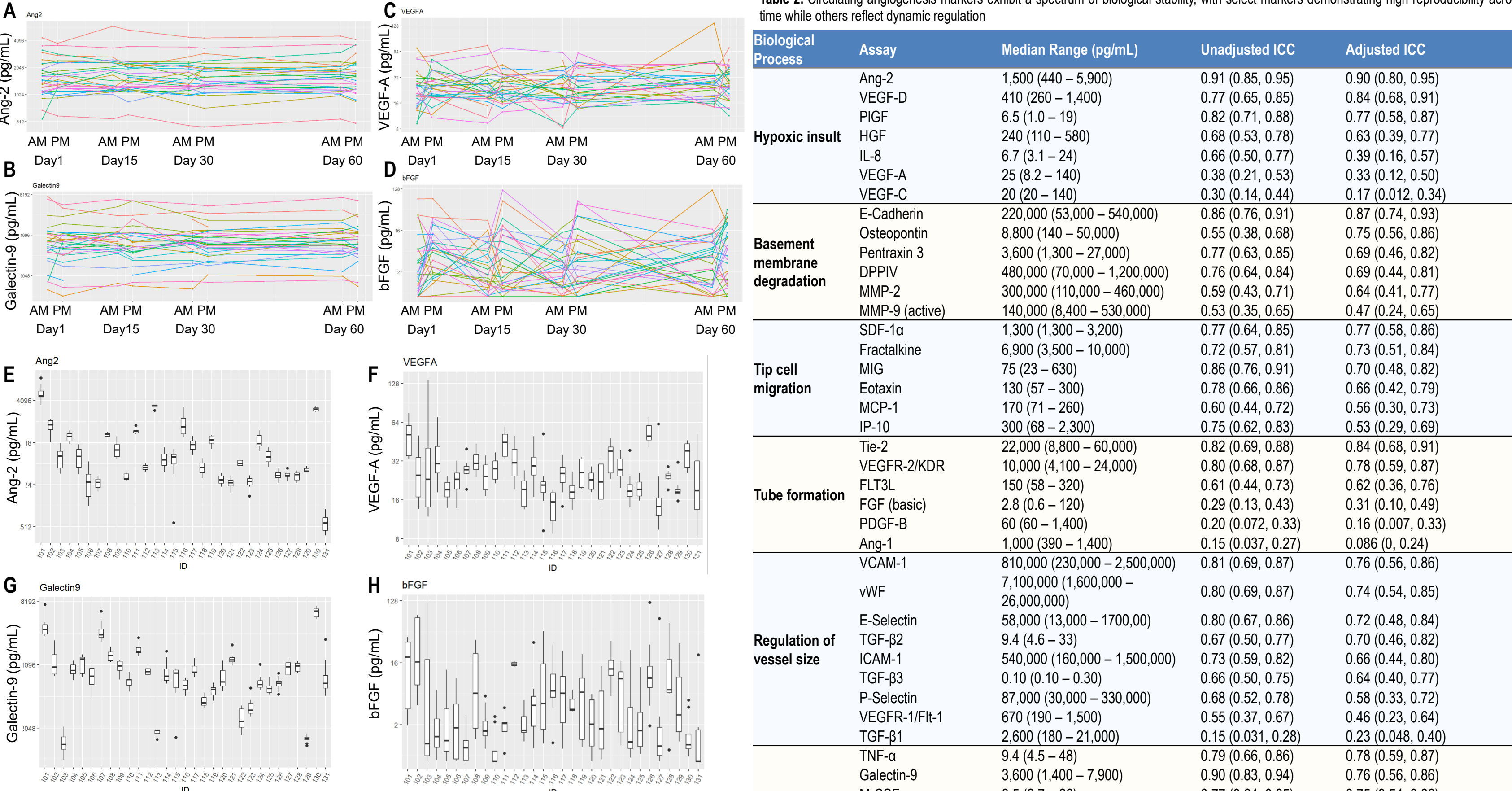
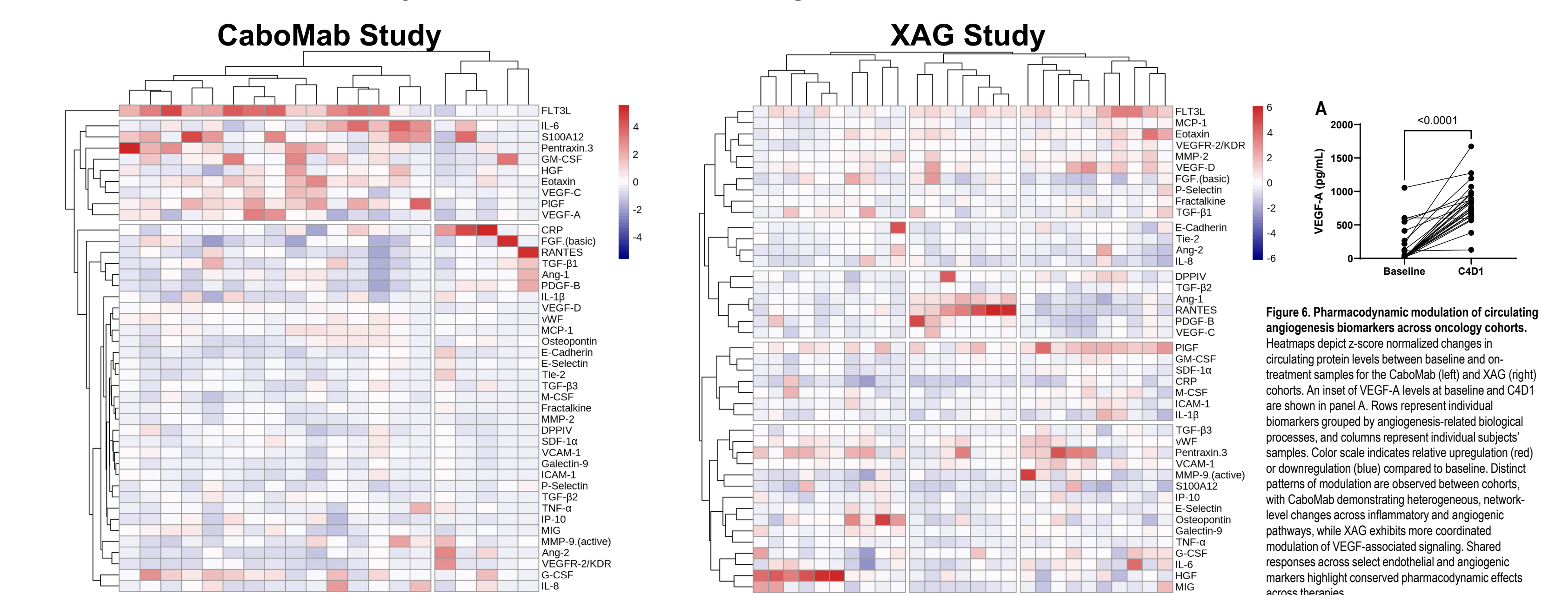


Figure 5. Distinct longitudinal behavior of angiogenesis biomarkers reveals stable versus dynamically regulated biology. (A-D) Scatterplots show individual longitudinal trajectories for Angiopoietin-2 (Ang-2), VEGF-A, Galectin-9 (Gal-9), and bFGF, respectively. (E-H) Corresponding boxplots summarize subject-level distributions across timepoints for each marker. Across markers, differences in trajectory, consistency, and distributional spread highlight varying degrees of longitudinal stability and intra-individual variability. Stable markers exhibit tightly clustered trajectories and minimal distributional shift over time, whereas more dynamic markers show increased dispersion and temporal fluctuation. These visual patterns align with adjusted ICC-derived stability metrics, supporting the distinction between consistently expressed biomarkers and those influenced by underlying physiological or temporal factors.

- Markers with high unadjusted ICC (e.g., Ang-2, VCAM-1, SDF-1 α) demonstrate robust longitudinal stability, supporting their use for baseline stratification and cross-sectional comparisons without adjustment.
- Markers that increase upon covariate-adjusted ICC (e.g., ICAM-1, MIG, Eotaxin) reflect context-dependent regulation, indicating sensitivity to systemic physiological state and the need for controlled sampling or covariate-aware interpretation.
- Markers with consistently low ICC in both unadjusted and adjusted models (e.g., VEGF-A, VEGF-C, PDGF-B, Ang-1) exhibit true biological variability independent of covariates, consistent with active involvement in dynamic angiogenic processes and suitability as pharmacodynamic biomarkers.
- Together, integration of unadjusted and adjusted ICC defines a functional biomarker framework, distinguishing robust baseline markers, covariate-sensitive markers requiring contextual interpretation, and intrinsically dynamic markers that may capture drug-induced biological change.

Network-Level Pharmacodynamic Modulation of the Angiome



- Conserved vascular response:** Both regimens modulate core angiogenic pathways (VEGF/endothelial signaling), indicating shared pharmacodynamic effects on tumor vasculature.
- Distinct response architectures:** CaboMab treatment exhibited a broad, immune-integrated remodeling signature, whereas XAG treatment exhibited a more focused, VEGF-axis-dominant response.
- Therapy-specific biology:** These patterns highlight how different treatment mechanisms shapes angiogenic network behavior—from diffuse, inflammation-linked signaling to coordinated vascular targeting.

Integrated Angiome Profiling Enables Translational Insight Into Vascular Biology and Tumor Angiogenesis

- A validated 44-plex platform delivers precise, high dynamic range quantification of angiogenesis biomarkers, supporting scalable profiling from discovery through translational research applications.
- Biomarker classification into stable and dynamic classes, combined with oncology cohort analysis, reveals therapy-specific angiogenic response architectures and supports baseline characterization and pharmacodynamic assessment.

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