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High-throughput screen identifies metabolite regulators of endothelial function

Fabiana Oliveira¹, Olga Bondareva¹, Anne Hoffmann¹, Florian Geier¹, Anastasia Kyselova², Christina Karantanou², Sofia-Iris Bibli^{2,3,4} & Bilal N. Sheikh^{1,5}✉

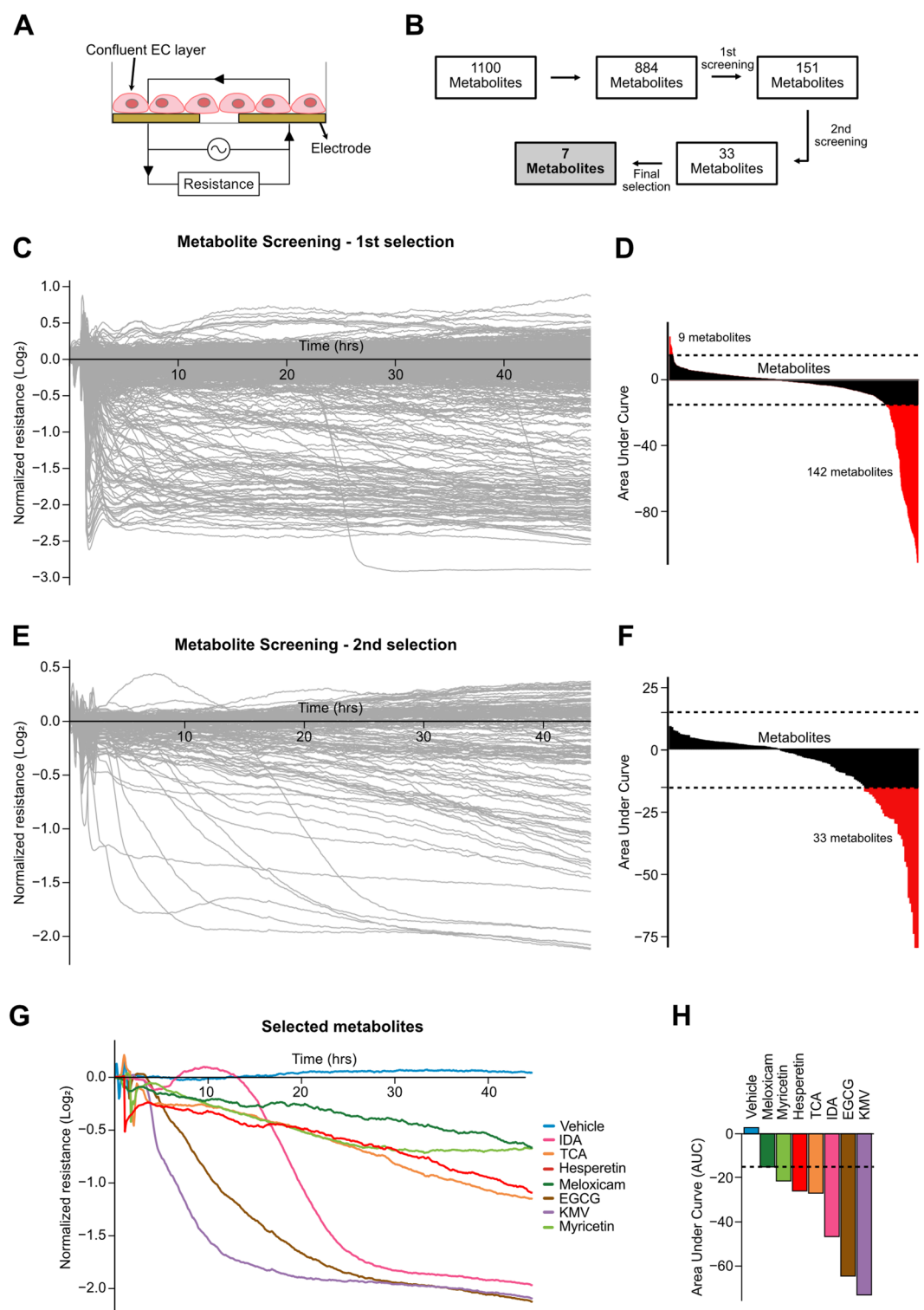
Endothelial cells (ECs) line blood vessels, regulate organ homeostasis, and their dysfunction is central to the development of cardiovascular diseases. Metabolites are critical regulators of EC health and function, yet only a limited number of metabolites that impact ECs have been identified. Here, we undertook a high-throughput screen of 884 metabolites and common drugs found in human biofluids to systematically identify compounds that impact barrier function in primary human umbilical vein ECs (HUVECs). Seven metabolites that deregulate vascular barrier function without causing cell death were shortlisted. These include (-)-epigallocatechin gallate, (±)-3-methyl-2-oxovaleric acid, taurocholic acid, myricetin, meloxicam, hesperetin and trans-3-indoleacrylic acid. These metabolites elicited differential effects on HUVECs, coronary artery ECs and pulmonary ECs. Transcriptional profiling revealed that each of the seven metabolites triggered distinct transcriptional responses in ECs, suggesting distinct mechanisms triggering EC barrier disruption. Notably, trans-3-indoleacrylic acid deregulated VE-cadherin junctions and increased EC sprouting, suggesting a potential role in angiogenesis. Together, these results provide a screening resource for metabolite-endothelial interactions and identify candidate circulating metabolites that regulate endothelial function.

Keywords Vascular permeability, Endothelial dysfunction, Metabolism, Metabolites, Angiogenesis

Endothelial cells (ECs) form the inner layer of blood vessels, where they function as a semi-permeable barrier between the bloodstream and organ parenchyma^{1,2}. ECs are metabolically active cells that dynamically regulate vascular homeostasis^{1,3–5}, and their dysfunction has been implicated in major diseases including dementia^{6,7}, coronary artery disease^{8,9}, stroke¹⁰, non-alcoholic fatty liver disease¹¹ and chronic heart failure¹². ECs display highly specialised phenotypes and expression profiles across organs, and contribute to essential processes such as angiogenesis^{13,14}, immune cell trafficking¹⁵ and the regulation of vascular permeability^{16,17}. Disruption of EC barrier integrity, often triggered by inflammatory or metabolic cues, leads to the dismantling of adherens and tight junction complexes and the subsequent formation of intercellular gaps^{16,17}. The resulting vascular leakage is a hallmark of multiple diseases including atherosclerosis¹⁸, pulmonary oedema¹⁹ and Alzheimer's disease²⁰. Therefore, identifying factors that drive endothelial barrier dysfunction is critical for understanding mechanisms driving a range of diseases underpinned by vascular dysfunction.

Metabolic stress is a major driver of endothelial dysfunction and cardiovascular disease^{21–25}. Recent metabolomics analyses of human cohorts have revealed that circulating metabolites can be strong predictors of cardiovascular disease risk in humans^{26,27}, acting not only as biomarkers but also as active contributors to vascular dysfunction. For example, high niacin levels increase EC inflammation and are associated with increased risk of cardiovascular events²⁸. Similarly, the gut microbiota-derived metabolite trimethylamine N-oxide (TMAO) promotes atherosclerosis and elevates cardiovascular disease risk^{29–31}. Another microbiota-derived metabolite, imidazole propionate, promotes atherogenesis by activating inflammatory pathways³². These observations illustrate the concept that individual metabolites can directly influence vascular function and cardiovascular disease risk, suggesting additional mechanisms remain undiscovered. Notably, there have been no unbiased, systematic screens of circulating metabolites to identify those impacting EC function.

¹Helmholtz Institute for Metabolic, Obesity and Vascular Research (HI-MAG) of the Helmholtz Center Munich, Leipzig, Germany. ²European Center for Angioscience, Department of Vascular Dysfunction, Medical Faculty Mannheim, Heidelberg University, Mannheim, Germany. ³German Centre for Cardiovascular Research (DZHK), Berlin, Germany. ⁴Helmholtz-Institute for Translational AngioCardioScience (HI-TAC) of the Max Delbrück Center for Molecular Medicine in the Helmholtz Association (MDC), Heidelberg University, Heidelberg, Germany. ⁵Faculty of Medicine, University of Leipzig, Leipzig, Germany. ✉email: bilal.sheikh@helmholtz-munich.de



In this study, we performed a large-scale screen of circulating metabolites to identify compounds that disrupt EC barrier integrity. We hypothesized that among circulating metabolites and compounds present in human biofluids, a subset would directly disturb endothelial barrier integrity. We systematically screened 884 metabolites for their effects on endothelial barrier integrity in human umbilical vein endothelial cells (HUVECs). Through sequential screening, viability assessment and independent validation, we shortlisted seven candidate metabolites which consistently disrupted EC barrier integrity without causing cell death. The seven metabolites triggered distinct transcriptional changes in HUVECs, suggesting unique candidate mechanisms that promote EC barrier breakdown. Our work provides a resource mapping the action of 884 metabolites on EC barrier integrity and establishes a framework for future mechanistic studies of metabolite-endothelial interactions.

◀ **Fig. 1.** Screening of metabolites that disrupt the endothelial barrier. **A** Schematic outlining the electrical cell-substrate impedance sensing instrument (ECIS). ECIS measures electrical resistance across the EC layer as a proxy for barrier function. **B** Metabolite screening flowchart outlining the step-by-step process leading to the final selection of 7 metabolites. **C** Normalized resistance of ECs over time for the first screening. Each line represents a different metabolite treatment ($n = 884$ different metabolites). **D** Area under the curve (AUC) calculation of endothelial barrier measurements (shown in **C**). Each vertical line represents a single metabolite treatment. Red lines represent metabolites selected for further testing (AUC values of < -20 or > 20). **E** Normalized resistance of ECs treated with 151 sub-selected metabolites for the secondary screen. **F** AUC calculations for the secondary metabolite screen (shown in panel **E**). Each vertical line represents a single metabolite treatment, averaged across 3 technical replicates. Red lines represent metabolites selected for continued experimentation (AUC values of < -15). **G** Normalized resistance of ECs treated with the seven selected metabolites: (-)-epigallocatechin gallate, myricetin, hesperetin, ($\pm$)-3-methyl-2-oxovaleric acid, meloxicam, taurocholic acid and trans-3-indoleacrylic acid. Normalized resistance values were extracted from the first screening. **H** AUC calculations of endothelial barrier measurements (shown in **G**). Colours represent different treatments. ECs, endothelial cells; EGCG, (-)-Epigallocatechin gallate; KMV, ($\pm$)-3-Methyl-2-oxovaleric acid; TCA, taurocholic acid.

Results

A high-throughput metabolite screen identifies compounds that disrupt EC barrier function

To identify circulating metabolites that directly impact endothelial barrier function, we performed a comprehensive metabolite screen. We utilised the complete MetaSci metabolite library, which contains 1100 compounds previously identified in human biofluids. The library included a wide range of compounds such as common byproducts of metabolism, molecules of plant origin, medicaments and others (Supplementary Table 1). Of these, 884 metabolites were soluble in solvents suitable for cell culture and were used for screening (Fig. 1B–C, Supplementary Table 2, 3). While concentrations of different metabolites vary in vivo, several endogenous metabolites occur in human plasma or serum at concentrations in the micromolar to millimolar range under physiological or pathophysiological conditions^{33,34}. Thus, 200 μ M was chosen as a standardized initial screening condition to enable comparison across a chemically diverse library, with follow up studies to more precisely define physiological relevance for sub-selected metabolites.

Endothelial barrier function was quantified in real-time for 48 h using the electrical cell-substrate impedance sensing instrument (ECIS)³⁵, which measures EC monolayer resistance as a proxy for barrier integrity (Fig. 1A)^{35,36}. The sensitivity of the assay was validated using thrombin, a known inducer of acute endothelial barrier disruption³⁷ (Supplementary Fig. 1A). The primary screen was performed as a staged workflow consisting of an initial single concentration screen, a secondary screening round with multiple technical replicates, viability-based exclusion of cytotoxic compounds, and independent validation of selected hits (Fig. 1B). Barrier function was quantified by calculating the area under the curve (AUC) values from normalised resistance measurements for each metabolite.

In the first screening round (Supplementary Table 2, 3), metabolites producing AUC values lower than -20 or above $+20$ were selected for further evaluation (Fig. 1B–D), resulting in 151 candidates. In the secondary screen, 33 metabolites consistently exhibited strong barrier-disrupting activity, defined by AUC values below -15 (Fig. 1E–F, Supplementary Table 4). These candidates were further refined based on cell viability and their biological significance. Seven metabolites emerged as robust disruptors of EC barrier integrity without impacting cell death: trans-3-indoleacrylic acid (IDA), taurocholic acid (TCA), hesperetin, meloxicam, (-)-epigallocatechin gallate (EGCG), ($\pm$)-3-methyl-2-oxovaleric acid (KMV), and myricetin (Fig. 1G–H). Reported human serum concentrations of these metabolites are summarized in Table 1.

Diverse metabolites impact EC barrier function without causing cell death

To ensure the robustness of our data, we undertook three independent ECIS experiments treating different batches of HUVECs with the seven shortlisted metabolites. Each of the seven selected metabolites significantly reduced HUVEC barrier resistance compared with vehicle-treated controls, accompanied by corresponding decreases in AUC values (Fig. 2A–U). Importantly, none of the seven selected compounds caused a reduction in cell viability, as determined by propidium iodide staining and flow cytometry (Fig. 2, Supplementary Fig. 1B), confirming that loss of barrier integrity was not secondary to cytotoxicity.

The seven shortlisted metabolites represent diverse biological classes, from plant flavonoids (EGCG, hesperetin, myricetin) to a bile acid (TCA), a branched-chain amino acid intermediate (KMV), an anti-inflammatory drug (meloxicam), and a gut microbiota-derived indole (IDA). This highlights the functional breadth of molecules capable of modulating endothelial barrier integrity.

Metabolite effects on endothelial barrier integrity differ across endothelial cell subtypes

Because ECs display tissue-specific phenotypes⁴⁶, we next evaluated whether the selected metabolites similarly disrupted barrier integrity in human coronary artery endothelial cells (HCAECs) and human pulmonary microvascular endothelial cells (HPMECs) (Fig. 2V). EGCG, KMV and hesperetin promoted a pronounced disruption in endothelial barrier integrity in both HCAECs and HPMECs (Supplementary Fig. 1C–H). Meloxicam, however, impaired the barrier integrity only in HCAECs (Supplementary Fig. 2A–B). IDA and TCA did not significantly affect HCAECs but damaged the barrier integrity in HPMECs (Supplementary Fig. 2C–F). Myricetin did not alter barrier integrity in either HCAECs or HPMECs (Supplementary Fig. 2G–H). These results

Metabolite	Control population	Control serum conc. (μM)	Disease	Disease serum conc. (μM)	Notes
trans-3-Indoleacrylic acid (IDA)	Healthy female volunteers ³⁸	24.00	Endometrial cancer ³⁸	46.00	–
Taurocholic Acid (TCA)	Healthy volunteers ^{39,40}	0.01–0.05	Drug-Induced Liver Injury ⁴¹	19.00	Strong elevation in pathology
Hesperetin	Healthy female volunteers ⁴²	2.42	–	–	5 h after 300 ml blood orange juice
Meloxicam	Healthy volunteers ⁴³	2.50–5.50	–	–	Oral administration of 7.5 or 15 mg/day
(-)-Epigallocatechin gallate (EGCG)	Healthy fasting volunteers ⁴⁴	0.20	–	–	1.5 h post oral intake
(±)-3-Methyl-2-oxovaleric acid (KMV)	Patients without heart failure ⁴⁵	5.20	Heart failure	19.30	–
Myricetin	–	Not reported	–	–	Limited human serum data

Table 1. Reported serum concentrations of selected metabolites in control (healthy) individuals and disease conditions. Concentrations were compiled from published studies and expressed in μM. Peak plasma concentrations following oral intake are provided, as specified in the original studies. Disease concentrations refer to reported levels in patient cohorts. Reported values may vary depending on analytical methods and sampling conditions.

demonstrate that metabolites can elicit differential responses across endothelial cell subtypes, emphasising the differential vulnerability of endothelial subtypes to deregulated metabolite levels.

Metabolite treatments trigger diverse transcriptional changes in ECs

To investigate the molecular changes underlying barrier disruption, we performed RNA-seq in HUVECs treated with each of the 7 selected metabolites. While all metabolites induced transcriptional changes in ECs, the magnitude varied greatly, with EGCG and hesperetin eliciting the most extensive changes in the HUVEC transcriptome (Fig. 3A, Supplementary Tables 5–11). Comparison of global transcriptional responses revealed limited overlap between metabolites. Although some pairs, such as KMV and EGCG, or KMV and TCA, showed moderately similar gene expression patterns, there was little correlation overall in the transcriptional changes induced by each compound (Fig. 3B). These findings indicate that each metabolite disrupts EC barrier integrity through a largely distinct set of molecular alterations.

We curated genes that show a similar response across the 7 metabolites. Analysis of the top 25 deregulated genes revealed the upregulation of the enzyme cyclooxygenase-2 (*PTGS2*) and the downregulation of the junction protein connexin 37 (*GJA4*) as a common feature across treatments (Fig. 3C). The list of top deregulated genes also included the non-coding RNAs *LINC01235*, *LINC02669* and *NORAD*. Common upregulated genes across treatments were associated with the HIF-1 and TNF signalling pathways (Fig. 3D, Supplementary Fig. 3A–G), suggesting the activation of stress and inflammatory signalling. Conversely, downregulated genes were associated with focal adhesions and actin cytoskeleton regulation (Fig. 3E, Supplementary Fig. 3A–G), which could trigger changes in EC barrier properties.

As junction proteins are essential for maintaining the endothelial barrier⁴⁷, we evaluated the expression of genes encoding EC junction proteins after treatment with each of the 7 shortlisted metabolites (Fig. 3F). The claudin family of proteins was broadly downregulated in EGCG-treated cells, with claudin 7 (*CLDN7*) most strongly downregulated following meloxicam treatment. In contrast, hesperetin triggered the upregulation of many claudin family members (Fig. 3F). Within the inflammatory class of molecules, EGCG, hesperetin and IDA triggered the upregulation of *ICAM1*, while *VCAM1* was specifically activated by hesperetin. The proinflammatory genes *CXCL8* and *IL11* were upregulated by myricetin (Fig. 3G). VEGF signalling is a key regulatory mechanism of endothelial homeostasis and permeability¹⁶; Hesperetin, meloxicam and EGCG caused an upregulation of *VEGFA* (Fig. 3H). Multiple metabolites including IDA, myricetin, meloxicam and hesperetin promoted the upregulation of oxidative stress associated genes including *NQO1*, *HMOX1*, *CYP1A1*, and *MT1X* (Fig. 3I), suggesting the activation of stress pathways.

Overall, these transcriptional data indicate that the selected metabolites disrupt EC barrier integrity through diverse and non-overlapping mechanisms.

Trans-3-Indoleacrylic acid (IDA) enhances sprout formation in ECs

To get a deeper look into mechanisms regulating EC barrier function, we focused on three metabolites identified in our screen: IDA (Fig. 2C), TCA (Fig. 2F) and myricetin (Fig. 2U). These metabolites were selected based on their pronounced effects on endothelial barrier function and their distinct biological relevance. To investigate molecular changes associated with loss of barrier integrity, we examined VE-cadherin junctional organization following metabolite treatment. Interestingly, IDA but not TCA or myricetin, significantly increased gap formation, as shown by VE-cadherin immunostaining (Fig. 4A), further highlighting metabolite-specific effects on EC junctional organization. These data suggest that altered VE-cadherin integrity may contribute to EC barrier disruption induced by IDA.

IDA is a tryptophan-derived metabolite synthesized by *Peptostreptococcus spp* in the human gut⁴⁸. Previous studies have reported beneficial vascular effects of IDA, including the rescue of vascular rarefaction and enhanced neovascularization in the skeletal muscle of aged mice⁴⁹. Given these reported vascular properties of

IDA, together with the pronounced disruption of barrier integrity observed in our screen (Fig. 2C), we examined the functional consequences of IDA on ECs in more detail.

We investigated the functional effects of IDA on endothelial barrier integrity by performing a concentration–response analysis. IDA induced a significant disruption in barrier integrity at 200 μ M, but lower concentrations had no significant effects (Fig. 4B). Notably, the concentration required to elicit barrier disruption (200 μ M) exceeds reported serum concentrations of IDA in both healthy individuals and disease states (Table 1).

We next investigated whether this loss of barrier integrity translated into increased endothelial permeability using a transwell assay. IDA significantly increased the permeability of the endothelial monolayer to a 3 kDa tracer after 24 hours of treatment, but no increase was observed after 4 hours (Fig. 4C). Together, these findings demonstrate that IDA disrupts endothelial barrier integrity in a concentration-dependent manner and impacts endothelial permeability with prolonged exposure.

We next evaluated whether IDA affects additional EC functions. In a spheroid angiogenesis assay, IDA treatment significantly increased the number and length of sprouts (Fig. 4D), consistent with a pro-angiogenic effect. However, we observed no IDA-mediated changes in a scratch-wound migration assay (Fig. 4E). Thus, IDA enhances EC sprouting without increasing migratory capacity in the scratch-wound assay.

As IDA-treated cells showed an upregulation of ICAM-1 (Supplementary Fig. 3I), we assessed whether IDA increased monocyte adhesion to HUVECs. IDA treatment for either 4 or 24 hours did not enhance the adhesion of THP-1 monocytes to HUVECs, whereas the TNF α -treated positive control showed a robust increase in monocyte adhesion (Fig. 4F). Thus, IDA impacts barrier function, VE-cadherin junctions and EC sprouting, without impacting monocyte adhesion or EC migration in a scratch-wound assay.

Discussion

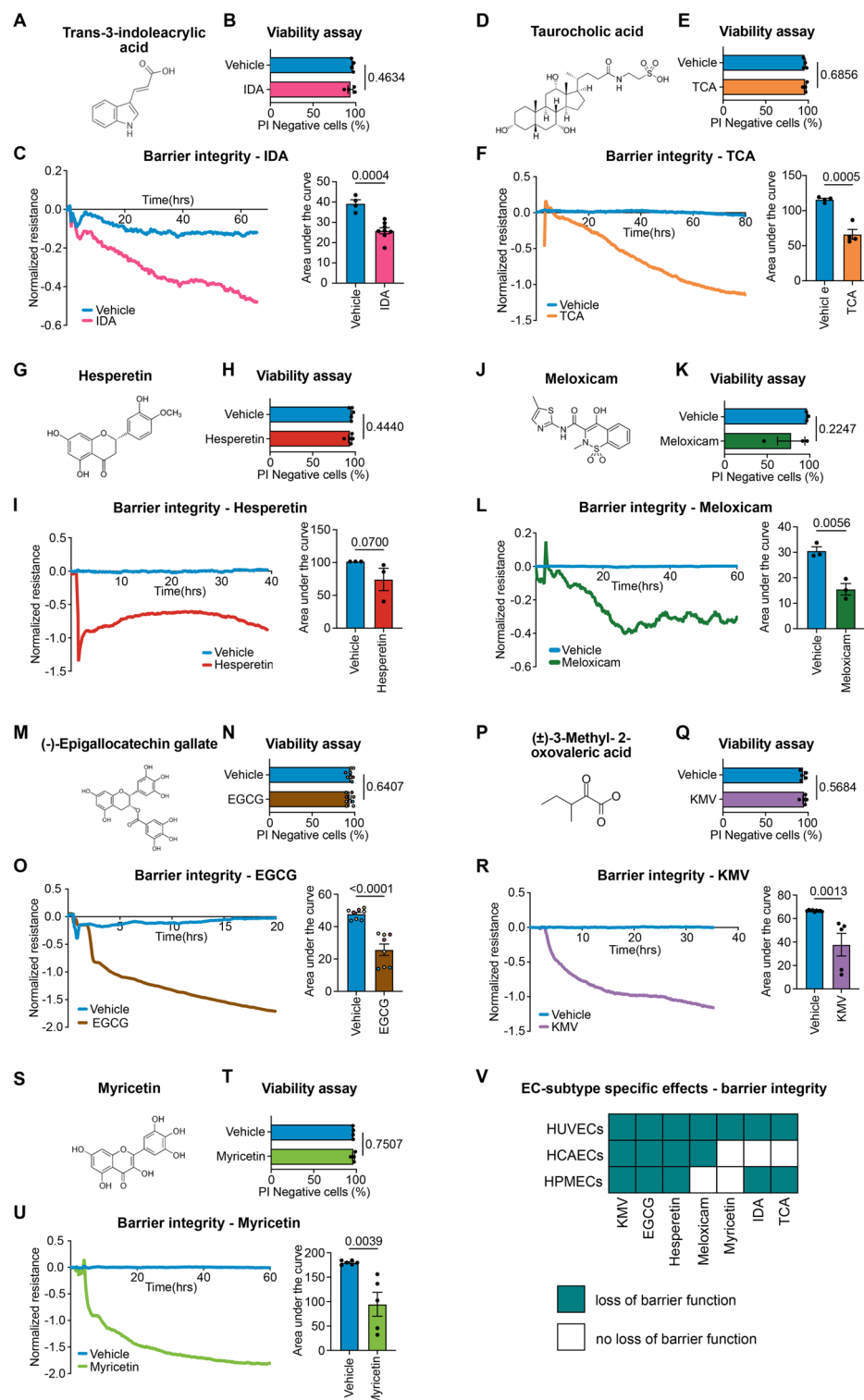
In this study, we performed an unbiased high-throughput screen of 884 metabolites to identify compounds impacting EC barrier integrity. We identified 7 metabolites that significantly impacted EC barrier integrity without causing cell death, including (–)-epigallocatechin gallate (EGCG), myricetin, hesperetin, ($\pm$)-3-methyl-2-oxovaleric acid (KMV), meloxicam, taurocholic acid and trans-3-indoleacrylic acid (IDA). Transcriptional analyses suggested that each metabolite promoted barrier dysfunction via distinct mechanisms. As an exemplary metabolite, we focused on IDA, which triggered defects in VE-cadherin junctions and promoted endothelial sprouting, with possible pro-angiogenic activity. Together, our work highlights that diverse metabolites can modulate EC function and provides a screening resource for future mechanistic studies.

The established association between metabolic risk factors and CVDs has fuelled efforts to identify individual metabolites that directly contribute to vascular dysfunction and disease progression. Through untargeted metabolomics, a wide range of compounds have been linked to increased CVD risk, including branched-chain amino acids^{50,51}, dietary metabolites^{52,53}, bile acids⁵⁴ and metabolites generated by the gut microbiome^{29–32,55}. These findings highlight the remarkable diversity of metabolic modulators that influence vascular and endothelial health, suggesting that many additional metabolites remain to be discovered and potentially leveraged as biomarkers or therapeutic targets. In this context, our work provides an important resource for the community, by systematically compiling the effects of 884 metabolites on EC barrier integrity.

In our study, the seven sub-selected metabolites showed differential effects on EC barrier function across endothelial cell subtypes. While metabolites such as EGCG, hesperetin and KMV impaired barrier integrity across multiple EC types, IDA and taurocholic acid selectively disrupted HUVECs and pulmonary ECs, but not cardiac ECs. These findings align with the concept that ECs display organotypic specializations, and organ-specific vulnerabilities to metabolic stress^{46,56}. For example, obesity induces distinct transcriptional programs across organs, including activation of inflammatory pathways in lung ECs, and an upregulation of lipid mobilization genes in liver ECs⁵⁷. Our study extends this concept by demonstrating that individual metabolites can drive subtype-specific EC responses, highlighting a potential mechanism through which metabolic risk factors promote EC subtype-specific pathologies. Nevertheless, the molecular mechanisms underlying these differential EC responses to metabolic stress remain poorly understood.

The seven selected metabolites induced distinct transcriptional responses, with limited overlap in global gene expression changes. Although partial similarities were observed among some metabolites, such as KMV with EGCG or TCA, the overall correlation across treatments was low, indicating that endothelial barrier integrity can be disrupted through multiple, metabolite-specific molecular routes. Pathways commonly upregulated included HIF-1 and TNF signaling, whereas downregulated pathways involved focal adhesion and cytoskeletal regulation, which are involved in maintaining barrier stability^{15,58–61}. Notably, the cyclooxygenase-2 enzyme (PTGS2/COX-2) was upregulated in six of the seven metabolite treatments. PTGS2 induction in endothelial cells has been associated with metabolic and oxidative stress responses^{24,62}, suggesting that its upregulation may reflect cellular adaptation to metabolite-induced stress. Since COX-2 catalyzes the production of prostaglandins, which can impact endothelial permeability and barrier disruption⁶³, COX-2-derived prostaglandin signaling may represent a common mechanism contributing to endothelial dysfunction observed across multiple metabolite treatments. At the gene level, changes in the expression of inflammatory mediators such as *ICAM1*, *VCAM1*, *CXCL8*, and *IL11*, as well as oxidative stress response genes such as *NQO1*, *HMOX1*, *CYP1A1*, *MT1X* were metabolite-specific. The transcriptomic changes also included non-coding RNAs, which may represent additional candidate mediators of metabolite-induced endothelial responses. Together, these findings suggest that circulating metabolites engage diverse signaling networks that converge functionally on regulation of endothelial function, while requiring targeted follow-up studies to establish direct mechanisms.

Among the selected metabolites, the gut microbiota-derived indole IDA⁴⁸ emerged as a particularly intriguing modulator of EC function. IDA disrupted barrier integrity and promoted loss of VE-cadherin junctional continuity, as observed by immunostaining. Together with the transcriptional downregulation of *CDH5*, these findings are consistent with a role for VE-cadherin in IDA-induced barrier disruption. Analysis of



VE-cadherin phosphorylation status in future studies could provide further insights into molecular mechanisms underlying these phenotypic changes. Moreover, IDA enhanced endothelial sprouting in a spheroid assay without affecting migratory capacity in the scratch-wound assay or monocyte adhesion, suggesting a potential role for this metabolite in angiogenic remodeling. However, as proliferation was not assessed in the current study, the cellular processes underlying IDA-induced sprouting remain to be determined.

Previous studies have identified IDA as an aryl hydrocarbon receptor (AhR) ligand with cytoprotective effects in epithelial or cancer contexts, and our data show induction of canonical AhR-associated targets such as *ALDH1A3*, *CYP1A1* and *CYP1B1* in ECs (Supplementary Table 5). AhR is highly active in pulmonary and intestinal ECs and plays a protective role in barrier integrity^{64,65}, suggesting that AhR may contribute to the effects of IDA on VE-cadherin disruption and endothelial sprouting. Future work is required to investigate this link. Considering the growing appreciation for microbiota-derived metabolites in cardiovascular disease,

Fig. 2. Selected metabolites disrupt endothelial barrier integrity while maintaining cell viability. **A** Molecular structure of trans-3-indoleacrylic acid (IDA) (CAS Number: 1204-06-4). **B** Flow cytometry quantification of viable cells after a 24-h IDA (200 μ M) treatment. Viable cells were defined as negative for propidium iodide staining. The associated FACS plots for each metabolite are provided in Supplementary Fig. 1B. **n** = 4 biological replicates per group. **C** Normalized endothelial resistance over time of HUVECs treated with IDA (200 μ M) or vehicle. Area under the curve (AUC) calculations of ECIS measurements are shown on the right. **n** = 4 biological replicates per group. **D** Molecular structure of taurocholic acid (TCA) (CAS Number: 145-42-6). **E** Flow cytometry quantification of viable cells after a 24-h TCA (200 μ M) treatment. **n** = 4 biological replicates per group. **F** Normalized endothelial resistance over time of HUVECs treated with TCA (200 μ M) or vehicle. AUC values are shown on the right. **n** = 4 biological replicates per group. **G** Molecular structure of hesperetin (CAS Number: 520-33-2). **H** Viability of HUVECs after a 24-h hesperetin (200 μ M) treatment. **n** = 5 biological replicates per group. **I** Normalized endothelial resistance over time of ECs treated with hesperetin (200 μ M) or vehicle. AUC values are shown on the right. **n** = 5 biological replicates per group. **J** Molecular structure of meloxicam (CAS Number: 71125-38-7). **K** Flow cytometry quantification of viable cells after a 48-h meloxicam (200 μ M) treatment. **n** = 3 biological replicates per group. **L** Normalized endothelial resistance over time of HUVECs treated with meloxicam (200 μ M) or vehicle. AUC values are shown on the right. **n** = 3 biological replicates per group. **M** Molecular structure of (-)-Epigallocatechin gallate (EGCG) (CAS Number: 989-51-5). **N** Viability quantification of ECs after a 24-h EGCG (200 μ M) treatment. Colours of the dots represent biological replicates (**n** = 4 per treatment). **O** Normalized endothelial resistance over time of cells treated with EGCG (200 μ M) or vehicle. AUC values are shown on the right. Colours of the dots represent biological replicates (**n** = 4 per treatment). **P** Molecular structure of ($\pm$)-3-Methyl-2-oxovaleric acid (KMV) (CAS Number: 66872-75-1). **Q** Flow cytometry quantification of viable cells after a 24-h KMV (200 μ M) treatment. **n** = 5 biological replicates per group. **R** Normalized endothelial resistance over time of HUVECs treated with KMV (200 μ M) or vehicle. AUC values are shown on the right. **n** = 5 biological replicates per group. **S** Molecular structure of myricetin (CAS Number: 529-44-2). **T** Viability of HUVECs after a 24-h myricetin (200 μ M) treatment. **n** = 4 biological replicates per group. **U** Normalized endothelial resistance over time of ECs treated with myricetin (200 μ M) or vehicle. AUC values are shown on the right. **n** = 5 biological replicates per group. **V** Schematic summarizing the endothelial subtype-specific effects of the selected metabolites on endothelial barrier integrity in HUVECs, human coronary artery ECs (HCAECs) and human pulmonary microvascular ECs (HPMECs). Filled boxes indicate metabolites that significantly disrupted barrier integrity in the respective primary endothelial cells. Detailed data and plots are provided in Supplementary Fig. 1 and 2. Data in bar graphs are represented as mean $\pm$ s.e.m. and were analysed with a two-sided Student's *t*-test by comparing the treatment groups to the appropriate vehicle (**B**, **C**, **E**, **F**, **H**, **I**, **K**, **L**, **N**, **O**, **Q**, **R**, **T**, **U**). AUC, area under the curve; CAS, Chemical Abstracts Service; ECs, endothelial cells; EGCG, (-)-Epigallocatechin gallate; HUVECs, human umbilical vein endothelial cells; IDA, trans-3-indoleacrylic acid; KMV, ($\pm$)-3-Methyl-2-oxovaleric acid; TCA, taurocholic acid.

IDA may represent a previously unrecognized candidate regulator of vascular homeostasis that warrants further investigation.

While this screen provides a valuable resource for evaluating metabolite–endothelial interactions, additional work is required to assess metabolite stability *in vivo*, bioavailability, and dose-dependent effects in endothelial cells. These properties may vary widely between metabolites. Of note, the 200 μ M screening concentration exceeds the circulating serum concentrations reported for several identified metabolites, including IDA. Future studies using tissue-specific concentration ranges for each metabolite will be important to more precisely define the pathophysiological relevance of these effects. Moreover, validation of barrier integrity changes using orthogonal permeability assays and further analysis of tight junction proteins will be required to determine if ECIS-associated changes detected here translate into altered vascular barrier permeability. Finally, *in vivo* studies will be essential to establish how these metabolites influence vascular function at an organismal level and across different vascular beds.

Together, through a high-throughput metabolite screen, we have identified circulating metabolites that disrupt endothelial barrier integrity. The 7 short-listed metabolites induced unique transcriptional changes, targeting different pathways and prompting subtype-specific EC responses. Amongst these, the gut microbiota-derived IDA emerged as a particularly interesting metabolite, due to its effects on EC barrier integrity and sprouting. Together, our work provides a resource covering metabolites that induce EC dysfunction and establishes a framework through which metabolite–endothelial interactions can be systematically explored in future mechanistic studies.

Methods

Metabolite library

The complete metabolite library (MetaSci) is comprised of 1100 metabolites and drugs found in human biofluids (Supplementary Table 1). All metabolites were dissolved in cell culture-compatible solvents: water, ethanol or dimethyl sulfoxide (DMSO). Of the original 1100 metabolites, 216 could not be dissolved and were removed from the screening. Metabolite stocks were further diluted in phosphate buffered saline (PBS) and screened at a final concentration of 200 μ M. For each compound, the corresponding solvent was included as control in all experiments. The 200 μ M concentration was used as a standardized screening condition across the library and was not intended to imply identical physiological exposure for all metabolites.

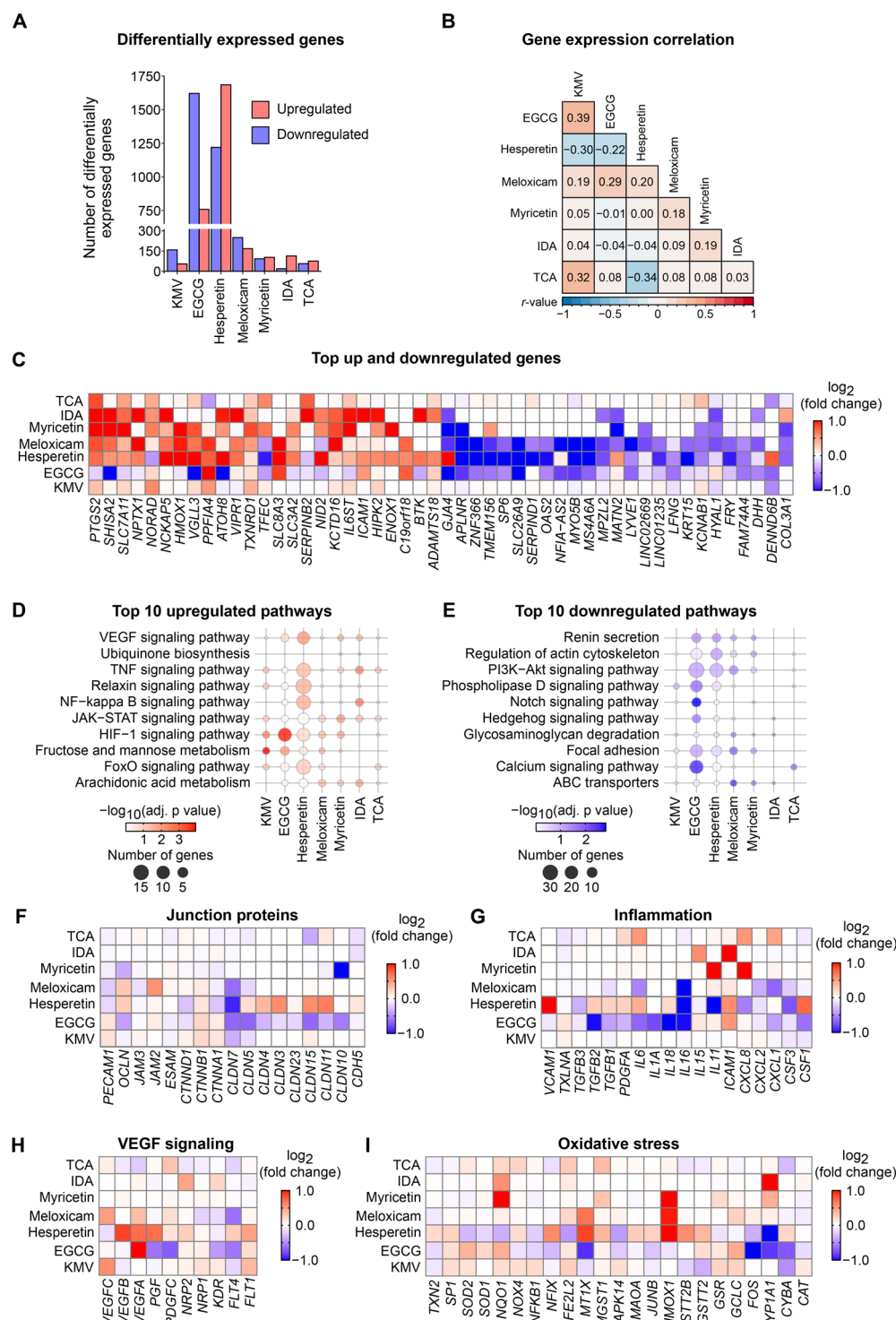


Figure 3

Cell culture

HUVECs (C-12200 and C-12203, PromoCell), HCAECs (C-12221, PromoCell) and HPMECs (C-12281, PromoCell) were cultured from passages 3 to 10 under physiologic normoxia at 3% O₂, since physiological levels of oxygen in the body are between 2–9%⁶⁶.

HUVECs were cultured on plates coated with 0.5% gelatine (48,723, Sigma-Aldrich) in endothelial cell growth medium (ECGM): Ham's F12 (21,765,037, Gibco) supplemented with 10% FBS (S0615, Sigma-Aldrich), 25 µg/ml Heparin (A3004, AppliChem PanReac), 1 µg/ml Ascorbic acid (A4403, Sigma-Aldrich), 2 mM GlutaMax (35,050,038, Gibco), 100 U/ml Penicillin–Streptomycin (15,140,122, Gibco) and Endothelial cell growth supplement (02–102, Millipore). HCAECs were cultured with MesoEndo cell growth medium (212–500, PromoCell). HPMECs were cultured with Endothelial Cell Growth Medium MV (C-22020, PromoCell).

◀ **Fig. 3.** Selected metabolites trigger non-overlapping transcriptional changes in ECs. **A** Number of differentially expressed genes in HUVECs after treatment with the indicated metabolites. Differentially expressed genes were defined by p -value < 0.05 ; $\log_2$ fold change > 0.5 or < -0.5 . **B** Correlation matrix comparing differentially expressed genes in HUVECs treated with the selected metabolites. Pearson correlation coefficients (r -values) are provided. Differentially expressed genes induced by at least one metabolite were collated for these analyses. **C** Heatmap of top 25 most consistently upregulated (red) and top 25 most consistently downregulated (blue) genes in ECs treated with the 7 selected metabolites. **D–E** KEGG-annotated pathways^{74–76} (**D**) upregulated and (**E**) downregulated in HUVECs after treatment with the 7 sub-selected metabolites. Enrichment analysis was performed separately for each condition and the most common pathways across all treatments were selected. **F–I** Expression of genes related to (**F**) junction proteins, (**G**) inflammation, (**H**) VEGF signalling and (**I**) oxidative stress in ECs treated with the selected metabolites. Data were derived from RNA-seq, $n = 4$ replicates per group. ECs, endothelial cells; EGCG, (-)-Epigallocatechin gallate; HUVECs, human umbilical vein endothelial cells; IDA, trans-3-indoleacrylic acid; KMV, ($\pm$)-3-Methyl-2-oxovaleric acid; TCA, taurocholic acid; VEGF, vascular endothelial growth factor.

Electric cell-substrate impedance sensing assay

ECIS 96W10idf plates (Applied Biophysics) were coated with 0.5% gelatine (48,723, Sigma-Aldrich) for 30 min (HUVECs) or left uncoated (HPMECs and HCAECs), followed by an overnight incubation with 100 μ l DMEM (41,966–029, Gibco). The cysteine present in DMEM contributes to the stabilization of the electrodes in the ECIS plate by forming covalent linkages with the gold. The following day, the DMEM was aspirated and 100 μ l of fresh media was added. The coated plate was placed on the 96-well station attached to the ECIS® Z-Theta instrument (Applied Biophysics). A rapid stabilization was performed. Next, 10,000 cells were seeded per well, making the total media volume 200 μ l. HUVECs, HCAECs and HPMECs were used. The plate was incubated for 5 min at 37 °C to equilibrate the temperature and placed back on the ECIS station. Real time values of resistance, impedance and capacitance were measured at three different frequencies: 1000, 4000 and 60,000 Hz. Cells were grown until confluency, indicated by constant capacitance values (usually after 24 to 48 h). When cells reached confluency, metabolites were added (200 μ M). Resistance measurements were performed for at least 48 h after treatment. An individual experiment was performed with thrombin (1 U/ml) (VWR, 605,157–1) prior to the screening to evaluate the sensitivity of the assay. The primary screen was carried out as a single-concentration discovery screen, followed by secondary screening and subsequent validation of shortlisted hits in independent experiments using different HUVEC batches.

Impedance values were extracted using ECIS Software v1.2.254.06 (Applied Biophysics) and processed through a multi-step normalization pipeline. To minimize inter-plate variability, measurements were first normalized to the mean of control samples within each plate, then $\log_2$ -transformed. Data were subsequently baseline-normalized to the pre-treatment time point (1 h before treatment) by dividing each value by its corresponding – 1 h measurement, establishing a common starting point across all conditions.

Vehicle controls matched to the solvent of each compound (water, ethanol, or DMSO) were included on every plate, and AUC values were calculated relative to the corresponding plate-specific control group. For the initial metabolite screen, compounds with AUC values > 20 or < -20 were selected for further testing. In the secondary screen, compounds with AUC values < -15 were selected. Statistical comparisons between treatment and vehicle control groups (water, ethanol, or DMSO, as appropriate) were performed using two-sided Student's t -tests on AUC values.

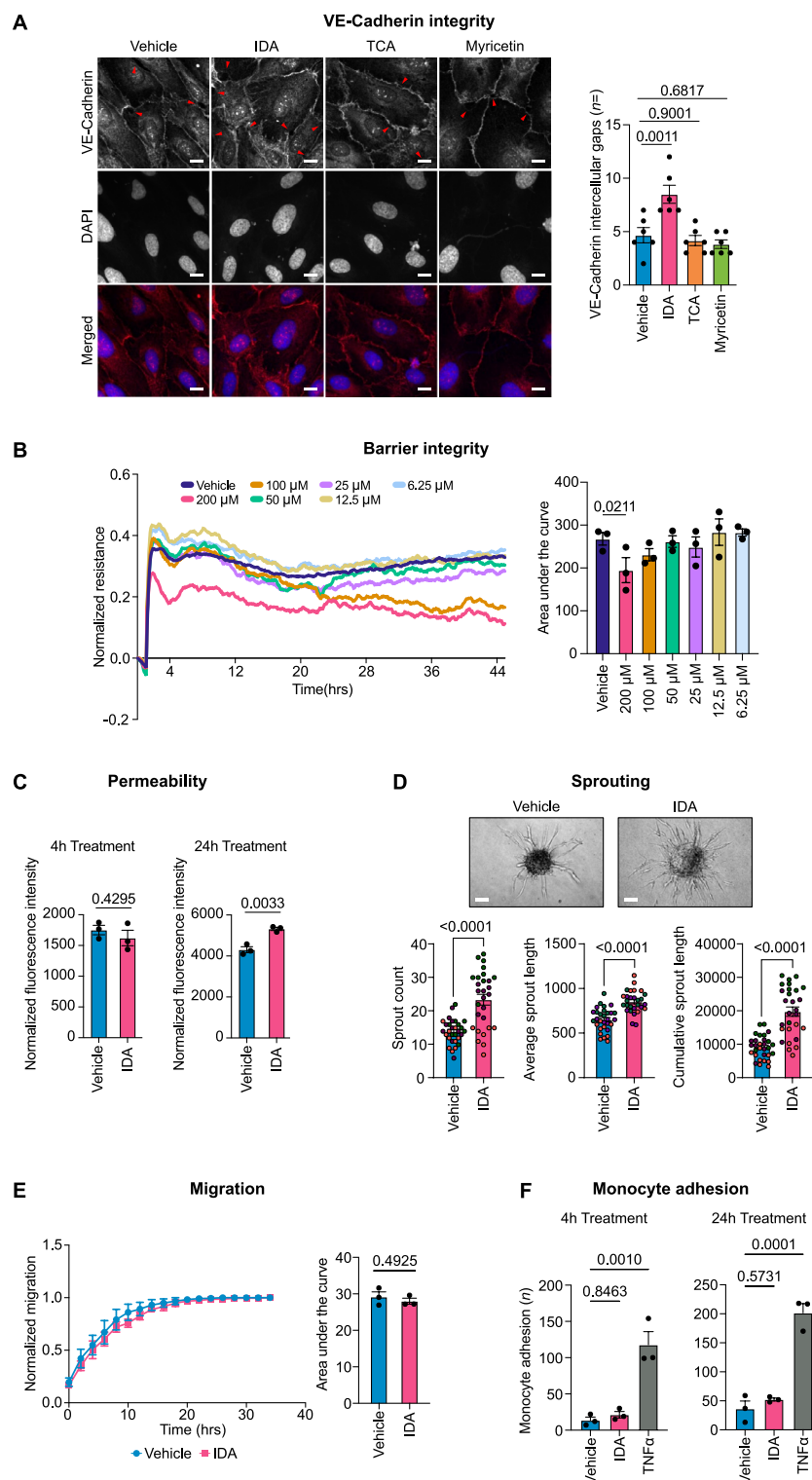
Bulk RNA sequencing and analysis

HUVECs were exposed to the selected metabolites for 4 h (KMV), 24 h (IDA, TCA, hesperetin, EGCG and myricetin) or 48 h (meloxicam). Cells were subsequently washed twice with ice-cold PBS, and total RNA was isolated using the miRNeasy Mini Kit (Qiagen, 217,004) in accordance with the manufacturer's instructions.

Library preparation and sequencing were carried out at the Genomics Core Facility of Helmholtz Munich. RNA quality was assessed by determining the RNA integrity number (RIN) with a LabChip GX Touch Nucleic Acid Analyzer (Revvity), and RNA concentration was quantified using Qubit. Samples with RIN values greater than 7 were used for poly(A)-enriched mRNA sequencing. Strand-specific libraries were generated using the Illumina TruSeq Stranded mRNA Library Preparation Kit following the manufacturer's protocol. After final quality control, libraries were sequenced on an Illumina NovaSeq 6000 platform using an S2.200 flow cell (2×100 bp paired-end reads), achieving a sequencing depth of at least 30 million reads per sample.

Raw reads underwent quality filtering and preprocessing with fastp (v0.23.3)⁶⁷, including adapter trimming, removal of low-quality bases, and elimination of poly-G artifacts. Read quality was further evaluated using FastQC (v0.11.4)⁶⁸. High-quality reads were aligned to the human reference genome (GRCh38.p13)⁶⁹ using STAR (v2.5.2b)⁷⁰ with default settings, allowing up to 50 multimapping alignments per read. Gene-level read counts were generated with featureCounts (v2.0.1)⁷¹ using parameters appropriate for reverse-stranded libraries. Reads mapping to multiple genomic locations were assigned fractionally to ensure accurate estimation of gene expression levels.

Differential gene expression analysis was conducted using the DESeq2 R package (v1.42.1)⁷². Genes were classified as upregulated or downregulated based on $\log_2$ fold change thresholds of > 0.5 and < -0.5 , respectively, with statistical significance defined as a p -value < 0.05 . Data were normalized using the variance stabilizing transformation (VST). Pathway enrichment analysis was performed with the EnrichR package (v3.2)⁷³, using the KEGG^{74–76} and BioPlanet⁷⁷ databases. Pearson correlation coefficients were calculated and visualized with



the corrplot package⁷⁸ (v0.95). Data visualization and additional plots were generated with ggplot2 (v3.5.1). All analyses were carried out in R (v4.3.0).

All sequencing data have been uploaded to NCBI GEO and are available under GSE332745.

Viability assay

HUVECs were cultured in 6-well plates, and cells treated for 24 h (IDA, TCA, hesperetin, EGCG, KMV and myricetin) or 48 h (meloxicam). After treatment, media from treated cells was saved into a collection tube. Cells were washed twice with PBS, and the discarded PBS was saved into the same collection tube. Cells were trypsinised and harvested into the same collection tubes, and centrifuged (300 $\times$ g, 5 min). The pellet was resuspended in 500 μ l 2% FBS (S0615, Sigma-Aldrich) and stained with 0.5 μ l 0.1 mg/ml propidium iodide (PI)

◀ **Fig. 4.** Trans-3-Indoleacrylic acid (IDA) impacts endothelial sprouting. **A** Representative VE-Cadherin immunostaining of HUVECs treated with IDA, TCA and myricetin for 24 h. In merged images, VE-Cadherin is shown in red and DAPI in blue. Scale bars indicate 10 μ m. Number (n) of gaps are shown on the right. Gaps were counted on 6 randomly chosen areas. **B** Normalized endothelial resistance over time of HUVECs treated with a concentration gradient of IDA (200, 100, 50, 25, 12.5 and 6.25 μ M) or vehicle. Area under the curve (AUC) calculations of ECIS measurements are shown on the right. n = 3 biological replicates per group. **C** Measurement of 3 kDa fluorescein-Dextran transport from the top to the bottom chamber in a Transwell permeability assay in HUVECs treated with IDA (200 μ M) or vehicle. Dots indicate biological replicates (n = 3 per treatment). **D** Endothelial cell sprouting in a modified spheroid assay. Spheroids were treated with vehicle or IDA. Scale bars indicate 100 μ m. Sprout count, average sprout length and cumulative sprout length are shown on the right. Dots correspond to data from individual spheroids, with colours of each dot representing biological replicates (n = 3 per treatment). **E** Scratch wound assay of HUVECs treated with IDA or vehicle. Migration of cells was followed by time-lapse microscopy. Area under the curve values are shown on the right. Dots indicate biological replicates (n = 3 per treatment). **F** Count of THP-1 monocyte adhesion to HUVECs treated with IDA, TNF- α or vehicle. Cells were pre-treated with vehicle and IDA for 4 or 24 h and with TNF- α for 30 min. Dots indicate biological replicates of HUVECs (n = 3 per treatment). Data are represented as mean $\pm$ s.e.m. and were analysed with a (**A**, **B**, **F**) one-way ANOVA test or (**C**, **D**, **E**) two-sided Student's *t*-test. HUVECs, human umbilical vein endothelial cells; IDA, trans-3-indoleacrylic acid; TCA, taurocholic acid; TNF- α , tumor necrosis factor alpha.

(P4864, Sigma-Aldrich). PI fluorescence intensity was measured with the BD LSR Fortessa instrument. Data were analysed with FlowJo™ 10.8.2. Cells with a strong PI signal were classified as dead.

Transwell permeability assay

HUVECs were seeded on 0.4 μ m Transwell inserts (CLS3472, Corning) at (100,000 cells/cm²) and incubated for 3 days. Treatment was added to the top chamber (250 μ l): IDA (200 μ M) or DMSO and Dextran Fluorescein 3000 MW (0.1 mg/ml; D3306, Invitrogen). Media was changed on the bottom chamber (500 μ l). After 4 and 24 h, 100 μ l of the bottom chamber was transferred to a clear bottom 96-well plate. Samples were diluted with 100 μ l absolute ethanol and fluorescence was measured at 494 nm excitation and 521 nm emission. Background values were subtracted from all samples.

Immunostaining

HUVECs were cultured in 8-well Millicell EZ Slides (PEZGS0896, Millipore) and treated with IDA, TCA and myricetin (200 μ M) for 24 h. Cells were fixed with 200 μ l ROTI®Histofix (P087.1, Carl Roth) for 15 min and washed with PBS twice. The slides were permeabilized for 30 min with 0.1% Triton X-100 (3051.3, Roth) in PBS then blocked for 1 h with 5% donkey serum (S30-M, Sigma-Aldrich) in PBS. Cells were incubated overnight at 4 °C with anti-VE-Cadherin antibody (1:100) (ab33168, Abcam) in 0.1% Triton X-100 and 1% donkey serum, washed 2 times with PBS and incubated for 2 h with AlexaFluor 647 donkey anti-rabbit IgG antibody (1:200) (ab150075, Abcam) in PBS. Slides were mounted on Mowiol (0713.1, Roth) with DAPI (A3672, AppliChem PanReac) mounting media. Images were obtained with a confocal microscope (LSM980, Zeiss) and analysed with ImageJ. Intercellular gaps were identified as breaks in the continuous VE-cadherin signal along the cell perimeter.

Sprouting assay

The protocol was based on previous work⁷⁹. For this assay a Methocel stock solution was prepared with 1.6% methyl cellulose (M0512, Sigma-Aldrich) in EGM-2 media (C-22011, Promocell). 455,000 HUVECs were aliquoted in a final volume of 13.6 ml and 1.4 ml methocel stock solution was added. After careful mixing, the cell suspension was pipetted into several 25 μ l drops onto a 10 cm square Petri dish. The hanging drops were incubated upside down for 24 h in a cell culture incubator. Next, spheroids were harvested with 6 ml of 10% FBS in PBS and centrifuged at 200 xg for 8 min at room temperature. Meanwhile, the collagen solution was prepared on ice: 12.45 mM HEPES (H0887, Sigma-Aldrich), 32.4 mM NaHCO₃ (25,080–060, Gibco), 4.2 μ g/ml Rat Tail Collagen Type 1 (354,236, Corning) and ~4 mM NaOH. After centrifugation, the supernatant was aspirated and the spheroids were resuspended in 1.2 ml methocel stock solution. Rapidly, 1.2 ml of collagen solution was added to the spheroids and gently mixed. 300 μ l of the spheroid suspension was transferred to a 48-well plate and incubated for 30 min in a cell culture incubator for polymerization. EGM-2 media (100 μ l) was added on top of the gels with the treatments: 200 μ M IDA (sc-256519, Santa Cruz) and DMSO (A3672, AppliChem PanReac). Spheroids were incubated with treatments for 24 h in a hypoxia incubator (5% O₂, 5% CO₂, 37 °C). Gels were washed with PBS and fixed with 100 μ l ROTI®Histofix (P087.1, Carl Roth) for 1 h.

Spheroids were imaged using phase contrast microscopy. Individual sprouts were manually traced with ImageJ. Sprout lengths were corrected according to the microscope's resolution.

Endothelial migration

Three biological replicates of HUVECs (50,000 cells) were cultured in uncoated 8 chamber slides (80,806, Ibidi) until confluency. Cells were scratched longitudinally with a 200 μ l pipette tip. Chambers were washed with Dulbecco's PBS (14,040,141, Gibco) and treatments were added in full EGM-2 media: 200 μ M IDA (sc-256519, Santa Cruz) and DMSO (A3672, AppliChem PanReac). Cells were incubated in an IncuCyte imaging

system (Essen Bioscience) that took 16 photographs per chamber automatically every 2 h for 2 days. Endothelial migration was analysed with ImageJ.

Monocyte adhesion assay

50,000 HUVECs were cultured in 8-well μ -slides (Ibidi, 80,826) until confluency. Cells were starved for 4 h in 200 μ l FCS-free media, 0.1% BSA in EGM-2 (CC-3162, Lonza). Next, HUVECs were treated with 200 μ M IDA (sc-256519, Santa Cruz) and DMSO (A3672, AppliChem PanReac) in full EGM-2 (200 μ l) for 4 and 24 h, and with 10 ng/ml TNF α (300-01A, Thermo Scientific) for 30 min. Cells were washed with PBS and 100,000 THP-1 cells were added to each chamber. THP-1 cells are a human monocyte cell line^{80,81}. HUVECs and THP-1 cells were briefly co-cultured for 30 min to allow monocyte adhesion. Cultures were washed to remove suspended cells and fixed in 200 μ l ROTI[®]Histofix (P087.1, Carl Roth) for 15 min. Phase contrast microscopy pictures were obtained and the amount of adherent THP-1 cells per chamber was quantified manually.

Statistical analysis

Data were analyzed using GraphPad Prism v10.0. Data are presented as mean $\pm$ SEM. Data were analyzed using two-tailed Student's *t*-tests, one-way ANOVA and two-way ANOVA where applicable, as indicated in the figure legends.

Data availability

All sequencing data have been uploaded to NCBI GEO and are available under GSE332745.

Received: 25 May 2026; Accepted: 24 September 2026

Published online: 01 October 2026

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Acknowledgements

We thank Longsheng Liao, Jesús Rafael Rodríguez Aguilera and Rima Chakaroun for the helpful discussions. We are grateful to Katrin Jäger and Pia Glöckner from the flow cytometry core facility (University Leipzig), and to the Genomics core facility (Helmholtz Munich) for processing the RNA-seq samples. We thank Daniela Kern, Marlene Hausner and Claudia Gebhardt for technical help, and Konstanze Jülich-Gruner, Susanne Renno and Sandra Zentile for administrative support.

Author contributions

F.O. and B.N.S. conceptualized the project. F.O., O.B., F.G., A. K. and C. K. performed the experiments. A.H. did the bioinformatic analysis. S.I.B. and B.N.S. supervised the project and provided guidance. B.N.S. acquired funding. F.O. and B.N.S. wrote the paper. All authors revised the manuscript.

Funding

This work was supported by the Deutsche Forschungsgemeinschaft (DFG) project grants (457240345, 511049882), funding from the free-state of Saxony and Helmholtz Munich, the Initiative and Network fund of the Helmholtz Association, and the open access publishing fund of Leipzig University.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1038/s41598-026-73615-3>.

Correspondence and requests for materials should be addressed to B.N.S.

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