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Molecular heterogeneity in human stroke – What can we learn from the peripheral blood transcriptome?

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Abstract

Stroke is a multifaceted disease with genetic and environmental components like diet and lifestyle. The central nervous and immune systems display complex interactions, with the peripheral immune response participating in brain injury and repair mechanisms following stroke. The bidirectional communication between the injured brain and peripheral blood presents an opportunity to investigate the molecular changes in the latter. There is substantial heterogeneity in stroke pathogenesis, pathophysiology, comorbidities, and response to treatment and outcome. This is captured and underscored by heterogeneity in the peripheral blood transcriptome. The current review highlights the role of the human peripheral blood transcriptome architecture for molecular phenotyping of different stroke etiologies and comorbidities, and for identifying underlying molecular correlates with clinically important variables and outcomes. Specific transcriptome features can potentially provide targets for clinical translation and for prioritizing genes and pathways for evaluation in experimental models. We also propose an approach to study the patient-specific transcriptional architecture and uncover the combinatorial heterogeneity in altered pathways in stroke patients that can also guide the search for treatment and prevention targets. Deciphering the molecular heterogeneity of stroke in a tissue that can be easily accessed and monitored, such as peripheral blood, may improve clinical trial success.

Keywords

Stroke, hemorrhage, transcriptomics, gene expression, sex differences

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Rationale for studying the peripheral blood transcriptome in human stroke

Damaged brain cells after stroke release DAMPs (Damage Associated Molecular Patterns) which activate microglia and other cells to release inflammatory signals such as cytokines and chemokines. They signal to peripheral blood resulting in infiltration of leukocytes from blood to brain in a process called leukocyte extravasation.[1] Recruited neutrophils release MMPs and neutrophil extracellular traps, and together with other blood cell types also release reactive oxygen species and other neuroinflammatory molecules that damage the blood-brain barrier **(BBB)** (reviewed in[1]) Many leukocyte interactions are relevant to stroke, including leukocyte interactions with blood clots, platelets, atherosclerotic plaque, and endothelial cells through adhesion molecules.[2,3] Many studies have underscored the role of the peripheral immune and coagulation systems in brain injury and repair following stroke (reviewed in[4,5]). This bidirectional communication between the brain and the peripheral blood and the important systemic immune response following stroke (reviewed in[1,6–9]) provide an opportunity to study blood to investigate stroke biology and its molecular heterogeneity (Figure 1).

The transcriptome architecture in disease provides information on genetic, epigenetic, environmental and lifestyle influences on a condition using existing whole

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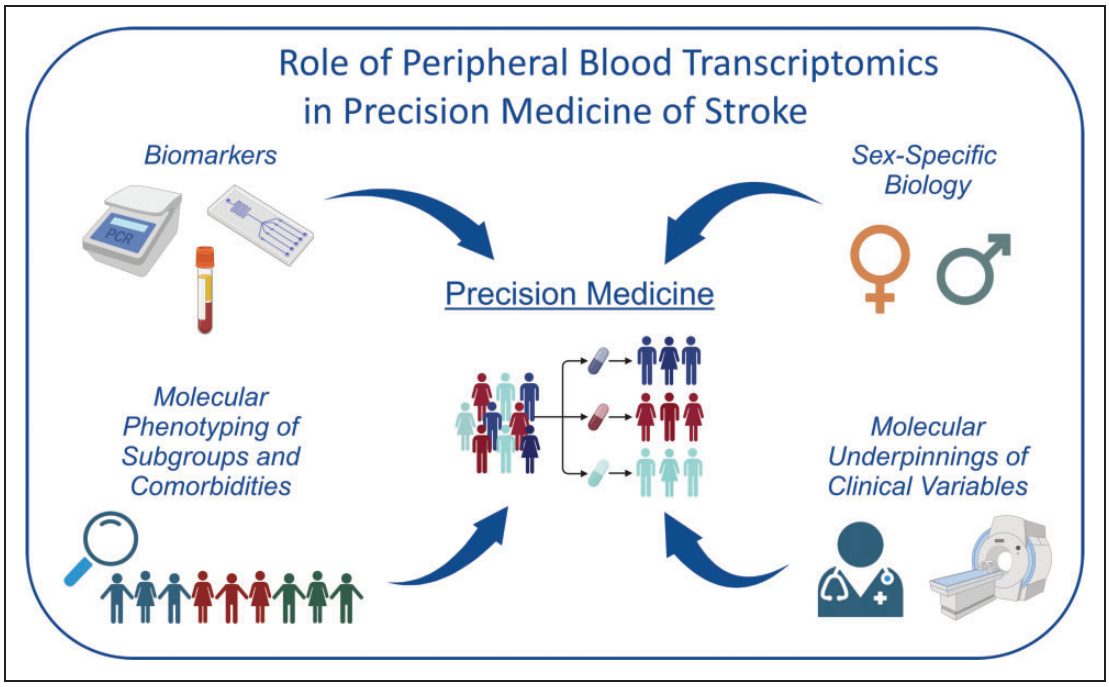

Figure 1. The role of peripheral blood transcriptomics in stroke precision medicine. Schematic presentation of the utility of the peripheral blood transcriptome for deciphering the sub-group heterogeneity in stroke for the advancement of precision medicine. Created with BioRender.com.

genome technologies that can investigate it in its entirety. Additionally, studies have revealed a significant overlap between differentially expressed genes in peripheral blood and in brain, such as for blood and brain in a middle cerebral artery occlusion nonhuman primate stroke model,[10] and in human peripheral blood[11] and perihematomal brain transcriptome[12] following intracerebral hemorrhage **(ICH)**. This suggests blood could serve as a surrogate for some changes in brain gene expression and could help identify treatment targets as well as monitoring effects of treatment.

Additionally, there is a need for human stroke transcriptomic studies because of the complex contribution of comorbidities to human stroke and because of difficulties in translating animal stroke studies to human stroke. For example, human and rodent peripheral blood cell composition differs significantly.[13] A mouse-human comparative peripheral blood transcriptomics study post stroke found little overlap in the affected pathways in the acute phase (9%) with more overlap in the subacute phase (47%).[14] This further underscores the need for human stroke molecular studies to help guide mechanistic animal stroke research with the overarching goal of improving translational success.

The transcriptional responses to ischemic stroke **(IS)**, spontaneous ICH, and subarachnoid hemorrhage **(SAH)** encompass dynamic coordinated shifts in transcriptional programs not only in brain cells, but also in peripheral blood cells.[15–18] Collectively, the human stroke transcriptomic peripheral blood studies demonstrate rapidly induced changes in the coding and non-coding transcriptome which are cell-type specific, time-dependent, and sex-specific, and reveal molecular heterogeneity in stroke subgroups.

Challenges exist in comparing omics studies, including transcriptomics studies. These challenges are due to employing different transcriptomic technologies (reviewed in[19]) and biological samples. The biological differences may relate to whether the transcriptomics studies are performed on whole blood, cell subpopulations, such as peripheral blood mononuclear cells (PBMC), individual cell subpopulations, or single-cell/single-nucleus RNA populations. Additionally, studies may differ due to sampling different time points. Moreover, variables such as treatment, infarct size, hemorrhage and edema volumes, cause and location, vascular risk factors, age, race, and sex, if not considered in the studies, may also affect the transcriptome findings.

The purpose of the review is to underscore the utility of the peripheral blood transcriptome to investigate the heterogeneity of the peripheral blood transcriptome following stroke to molecularly phenotype causes of stroke subtypes, to uncover biological networks underlying clinically relevant features of stroke, and to decipher the effects of comorbidities including sex-specific

biology. Thus, the present review highlights selected studies to illustrate what knowledge can be obtained from examining the peripheral blood transcriptome in human stroke. Due to differences in stroke populations, the evolution of molecular responses over time, cell-specific responses, and different technologies used to assess the transcriptome, direct comparison of findings among stroke transcriptome studies is challenging, as pointed out above, and not the focus of this review.

Stroke etiologies

Distinct transcriptional programs following ischemic stroke of different etiologies

IS has different etiologies including cardioembolic **(CE)**, large-vessel atherosclerotic **(LVA)**, lacunar (small vessel, **SV**), other causes, and undetermined (cryptogenic).[20] Identifying the IS cause is essential for optimal treatment and prevention strategies.[21] The heterogeneity in etiologies is also reflected in the composition of the thrombi, with CE stroke having less red blood cells and more fibrin/platelets than thrombi with non-CE origin.[22] Response to thrombolysis and thrombectomy treatment has also been found to be different based on thrombus composition with red blood cell-rich thrombi typically associated with more favorable outcomes, while fibrin-rich thrombi – with less favorable outcome (reviewed in[23]). The IS peripheral blood transcriptomic response is heterogeneous based on IS etiology[24–29] and has an etiology-specific time-course.[16] Profiles for CE, LVA, and SV etiologies have been developed,[24–27] and used to predict IS cause in cryptogenic IS, though larger validation studies are needed. Studies identifying biomarkers of IS etiology are reviewed in[30] and additional studies are underway[31] as identifying stroke etiology is of paramount significance for the prevention of recurrent stroke. Here we discuss the transcriptional heterogeneity as the identified genes/pathways may guide the search for etiology-specific treatment targets.

Xu et al[24] investigated the peripheral blood transcriptome of CE and LVA IS in serial blood draws at $\leq$3 h (before thrombolytic treatment), 5 h and 24 h (post thrombolytic treatment time-points) post *ictus*, and of healthy controls. The IS subjects were from the CLEAR trial (NCT00250991),[32] where each patient was treated with tPA with or without eptifibatide within 3 h post *ictus*. Most LVA differentially expressed genes were predicted to be expressed in platelets and monocytes and modulated hemostasis, while most CE differentially expressed genes were expressed in neutrophils and modulated immune responses to infectious stimuli.[24] A follow-up larger study also identified etiology-specific immune responses to CE and LVA IS.[27] CE IS was associated with renin-angiotensin, thrombopoietin and NF-kB signaling, as well as with genes implicated in the main cardiac CE IS causes- atrial fibrillation (CREM, SLC8A1, KNCH7, KCNE1), myocardial infarction (PDE4B, TLR2), and heart failure (MAPK1, HTT, GNAQ, CD52, PDE4B, RAF1, CFLAR, and MDM2).[27] LV IS associated with T cell, monocyte/macrophage and cardiovascular function pathways, such as T cell activation and regulation, CCR5 signaling in macrophages, relaxin and corticotropin releasing hormone signaling pathways, as well as atherosclerosis and BBB dysfunction genes (MMP9, FASLG, RAG1, TNF, IRAG1, and THBS1).[27]

SV stroke also showed specific peripheral blood transcriptomic response with pathways enriched in Monocyte and Leukocyte Activation and Recruitment, and Innate and Adaptive Immune Cell Communication biofunctions among others.[26]

IS etiology-associated heterogeneity in the peripheral blood transcriptome has also been investigated at the level of the alternatively spliced transcriptome. Alternative splicing is a genetically-regulated and environmentally-influenced process by which a single gene can produce multiple alternatively spliced isoforms which have specific functions in different cells, tissues, developmental stages, and disease states.[33] It is a major process that generates vast molecular diversity by which the ~20,000 protein-coding genes code for >250,000 RNA and protein isoforms. Dykstra-Aiello et al[29] examined the differential alternative splicing of 71 putative stroke/vascular risk factor genes expressed in peripheral blood of 245 IS and controls, and determined many had sex-specific and etiology-specific pattern of expression. This underscored the importance of considering both sex and etiology when novel treatments are developed.[29] This study also confirmed a previous pilot RNA-Seq study,[34] which showed etiology-specific alternative splicing/exon usage is involved in CE, LVA and SV IS mechanisms, and in ICH. Studying the differentially alternatively spliced genes/differential exon usage is a highly understudied area in stroke research that warrants further investigation as exon/transcript isoform resolution will likely be required to better understand stroke biology, and to develop novel treatments and biomarkers.

The above-mentioned studies have been performed in whole peripheral blood which contains different cell types with specific roles in IS. RNA-Seq studies in isolated monocytes and neutrophils from peripheral blood revealed cell type-specific- and etiology-specific gene expression[28] and time-dependent gene expression using self-organizing maps.[16] Though there was some overlap in differentially expressed genes and enriched pathways among the three IS etiologies, the majority of the differentially expressed genes and pathways were

etiology-specific.[28] Most of the self-organizing map time-dependent profiles that were similar between the three IS etiologies were enriched in etiology-specific pathways, signifying the temporal evolution of the peripheral blood transcriptomes following human IS to be etiology-specific. Furthermore, there were pathways which progressed in opposite directions over time. For example, in neutrophils, genes from the toll-like receptor **(TLR)** signaling pathway were down-regulated at $\leq$24 h vs controls and increased after 24 h in CE stroke, while in LVA they were progressively down-regulated at both time-points compared to controls. TLR signaling modulates NFkB signaling and has been investigated as a potential treatment target in cardiovascular disease.[35]

Human transcriptomics studies in IS of different etiologies supplement experimental findings, which may strengthen their clinical translation. They have shown whole-blood transcriptome and cell-type specific heterogeneity in IS etiology, which is important since each cell type has specific roles in the injury and repair mechanisms following IS.[1,9,36] Additionally, the etiology-specific temporal evolution may pinpoint etiology-specific treatment windows. The observed molecular heterogeneity in IS of different etiologies may have repercussions for treatment development and may require a search for novel etiology-specific therapies.

Molecular heterogeneity due to ICH etiology/location

Intracerebral hemorrhage (ICH) is caused by bleeding into the brain parenchyma. It is the second most common cause of stroke, accounting for 10–20% of all strokes, and has high short-term and long-term mortality up to 50% at 30 days.[37–41] The primary ICH etiologies in the aged population are cerebral amyloid angiopathy (CAA)[42] and chronic hypertension.[37] CAA is characterized by deposition of amyloid in the media and adventitia of cortical and leptomeningeal small vessels, including arterioles and capillaries,[42] and is diagnosed based on the Boston v. 2.0 criteria.[43]

To the best of our knowledge, there have been no studies of the peripheral blood transcriptome in human ICH stratified by ICH etiology in older adults. A study by Knepp et al[44] investigated the common and unique transcriptional changes in the peripheral blood transcriptome in deep ICH (usually due to chronic hypertension) and lobar ICH (usually due to CAA). The authors identified deep ICH- and lobar ICH-common pathways such as Autophagy, T Cell Receptor, Inflammasome, and Neuroinflammation Signaling. However, there was a substantial molecular heterogeneity in the transcriptome architecture in deep and lobar ICH, with Th2, Interferon, GP6, and BEX2 Signaling pathways unique to deep ICH, while Necroptosis Signaling, Protein Ubiquitination, and various RNA Processing terms were unique to lobar ICH. Additionally, amyloid processing pathways and suppression of several protein ubiquitination pathways and upregulation of ubiquitin inhibitors were also unique to the lobar ICH transcriptome. The authors suggested peripheral blood cells may participate in amyloid removal or leading to perivascular/vascular amyloid in lobar ICH due to CAA. The co-expression networks and/or the potential master regulators of the deep and lobar ICH responses also showed heterogeneity in their cell-specific landscape– with deep ICH enriched in neutrophil- and monocyte-specific genes, while lobar ICH was enriched in erythroblast- and T cell-specific genes.[44] The study also identified additional layers of heterogeneity discussed in the Heterogeneity in Common Pathways section below.

Some ICH causes in the younger population have also been investigated. Weinsheimer et al[45] studied the peripheral blood response to brain arteriovenous malformations (BAVM), an important ICH cause in young adults. The authors investigated the changes in the transcriptomes of ruptured and unruptured BAVM patients and healthy controls.[45] They found many of the ruptured-BAVM-related genes play a role in inflammatory processes, consistent with a role of inflammation in BAVM pathogenesis.[46] The findings suggested potential pathogenesis-related changes in the transcriptome for BAVM and/or BAVM rupture, which may help identify biomarkers or novel treatment targets.

Comorbidities

Stroke comorbidities, such as age, diabetes, hypertension, hyperlipidemia, smoking, and sex (discussed in a separate section below) increase stroke risk and worsen outcome (reviewed in[47,48]). The experimental stroke field recognizes that a critical factor for improving the clinical translation of findings is to address the presence of comorbidities in human stroke which often have not been represented sufficiently in preclinical research (reviewed in[18,49]). The landscape of stroke comorbidities in the peripheral blood transcriptome following human stroke is also largely unexplored. Some human stroke studies address the effect of comorbidities by including vascular risk factor-matched controls (**VRFCs**), increasing the probability that the observed peripheral blood transcriptome differences reflect the response to the stroke and/or interaction with the risk factors. However, in addition to that, molecular phenotyping of stroke subgroups stratified by presence or absence of a particular comorbidity is essential and may help identify genes and pathways

involved in each subgroup and potential novel subgroup-specific treatment targets. Below, the transcriptomic studies related to some of the stroke comorbidities are discussed.

Age

Age is a risk factor for stroke.[18] The immune system changes with age which includes increased inflammation (inflammaging), attenuated immune response, and immunosenescence, with differences between males and females.[50,51] Age-associated peripheral blood transcriptome changes following human IS have been investigated by Sykes et al,[52] where the authors identified age-associated genes in a derivation cohort (n = 94) and confirmed them in a validation cohort (n = 79) of IS patients. Sixty-nine adaptive immunity genes associated with age in IS, 49 of which were reported as age-associated in non-stroke studies as well.[52] Supporting evidence for age-associated molecular changes in stroke also came from a study by Soriano-Tárraga et al.[53] It compared stroke and control patients' epigenetic/biological age, which is a measure of DNA methylation commonly correlated with normal aging and can be accelerated in some diseases. They found stroke patients had an older biological age than controls, indicating biological age and epigenetic modifications may be a useful stroke biomarker. Nakaoka et al.[54] analyzed gene expression of aneurysmal domes from patients with SAH and unruptured aneurysms, and superficial temporal artery samples as controls. Using hierarchical clustering, they identified two subgroups of SAH subjects with statistically different ages, demonstrating differing gene expression patterns based on patient age. These findings further underscore the molecular heterogeneity in the peripheral blood transcriptome and the importance of future studies investigating age-associated changes as they may modulate age-related stroke risk and outcome.

Atrial fibrillation

Atrial fibrillation is a major risk factor for CE IS. Many IS subjects initially deemed to have cryptogenic etiology are later discovered to have paroxysmal atrial fibrillation. A study of the peripheral blood transcriptome of IS patients uncovered additional levels of transcriptional heterogeneity based on presence or absence of atrial fibrillation in CE IS patients.[27] The study discovered specific immune responses associated with atrial fibrillation in CE IS and developed a 37-gene profile which differentiated CE due to atrial fibrillation from nonatrial fibrillation causes with >90% sensitivity and specificity. Additionally, a 40-gene profile differentiated LVA from CE with >95% sensitivity and specificity on cross-validation. When these profiles were applied to patients with cryptogenic stroke, 17% were predicted to have LVA and 41% to be CE. Of the cryptogenic strokes predicted CE, 27% were predicted to have atrial fibrillation.[27] These findings require validation in larger cohorts, as biomarkers identifying atrial fibrillation in cryptogenic strokes is of critical importance for future stroke prevention.

Smoking

Smoking is a modifiable risk factor for stroke. A whole-blood transcriptome meta-analysis of 10,233 subjects of European ancestry identified signatures of current and former smokers which included activation of platelets, immune response and apoptosis.[55] The smoking associated signature had a significant association with SNPs associated with stroke (ALDH2; CAMTA1; SH2B3; TMEM116; ERP29).[55] Cheng et al.[56] analyzed IS and control subjects stratified into current or never-smokers. Genes associated with smoking in IS patients were involved in pro-inflammatory pathways which may contribute to brain injury and worsen stroke outcomes. The authors also identified 10 genes associated with smoking which were common in the IS and control groups (GRP15, LRRN3, CLDND1, ICOS, GCNT4, VPS13A, DAP3, SNORA54, HIST1H1D, and SCARNA6). They speculated these genes might be associated with stroke risk.[56] The authors reported several of these genes have been implicated in stroke, such as ICOS which affects outcome in experimental stroke[57] and CLDND1, which is a tight-junction protein, involved in cerebrovascular disease pathogenesis of stroke-prone spontaneously hypertensive rats.[58] Most of the smoking-associated genes in IS patients did not overlap with the ones in controls, suggesting unique interactions between smoking and ischemic stroke in peripheral blood cells.[56]

Cancer

Cancer increases stroke risk and comorbid cancer in stroke is a growing area of research as roughly 13% of IS patients have a prior cancer diagnosis,[59] and 2%–10% will be diagnosed with cancer within a year post stroke.[60] A pilot study by Navi et al.[61] identified distinct peripheral blood gene expression patterns in IS patients with and without comorbid cancer, cancer alone, and VRFCs. Cancer-Stroke unique pathways included several interleukin signaling pathways (IL-1, IL-10, and IL-12), T helper-cell (Th1, Th2, Th17) activity, phagosome formation, pattern recognition receptor, TREM1 signaling, and neuroinflammation signaling pathways.[61] Additionally, major

transcriptional regulators were differentially expressed between the cancer-stroke and stroke-only groups, some of which have been implicated in experimental stroke models or in stroke-associated processes. Those included CREB1 and SQSTM1. CREB1 was down-regulated in the cancer-stroke group versus the stroke-only group. CREB transcription factors modulate circuit plasticity and functional recovery after stroke; with increased CREB levels fostering stroke recovery whereas inhibiting CREB signaling hinders recovery.[62] SQSTM1 (p62) is a regulator of the hypoxia response, NF-kB and TNF signaling,[63] and was upregulated in the cancer-stroke group versus the stroke-only group.[61]

A follow up study by Knepp & Navi et al[64] investigated the peripheral blood transcriptome of both protein – coding mRNA and regulatory miRNA in Cancer-Stroke patients, stroke only, cancer only, and VRFCs. They also investigated the IS-mechanism-specific transcriptome architecture of cancer-stroke patients.[64] Activation of Coagulation and Activation of Blood Platelets Biofunctions were observed in IS patients with cancer versus without cancer, supporting a hypercoagulable state in stroke with comorbid cancer. There were also differences in the complement system, growth factor signaling, and immune/inflammatory pathways. Among the differentially expressed genes, enrichment with granulocyte–specific genes was observed in both the CE and non-CE stroke response in patients with cancer compared to VRFCs, while T-cell-specific genes were associated only with CE strokes. The IS cause in cancer patients remains undetermined (cryptogenic) in ~50% of patients, though a hypercoagulable state and CE etiology are often clinically suspected.[60] Indeed, based on a 15-gene panel, 11 of the 16 (69%) cryptogenic strokes with cancer were predicted to have CE etiology.[64] A separate 15-gene panel predicted the presence or absence of cancer in the IS subjects, which in a validation cohort classified correctly 81% of the stroke with cancer and 71% of the stroke-only subjects.[64] This suggests the possibility that peripheral blood biomarker panels could identify occult cancer in IS patients. These subgroup-specific molecular differences may indicate different treatment targets for each subgroup including the stroke subgroup with comorbid cancer.

Heterogeneity in response to treatment/clinical complications

A major goal of precision medicine is developing biomarkers to predict response to treatment to identify which patients are good candidates for a particular treatment and are likely to respond well, and which patients are likely not to respond or to develop side effects or complications. Using biomarkers to predict elevated risk of complications could guide eligibility for treatment or improve allocation of care to high-risk hospitalized individuals. Discovering biomarkers for responders and non-responders for new treatments could be used to stratify patients in later phases of clinical trials and increase the chances of their success. Though some stroke clinical trials include a search for biomarkers for response to treatment or for complications, many are focused on known potential biomarkers and do not include a comprehensive omics approach. Investigators and funding agencies should consider a whole transcriptome screen, wide proteome screen, and/or multi-modal biomarker screen to identify responders in Phase I or II trials. These responder profiles could be used in Phase III trials and be extended to the clinic for screening for those most likely to respond in a precision medicine treatment approach.

Hemorrhagic transformation following tPA treatment in ischemic stroke

Recombinant tissue-plasminogen activator (rt-PA) has been the main thrombolytic IS treatment. A study found transcriptome changes in the IS peripheral blood transcriptome PRIOR to tPA administration and predicted which patients will LATER develop hemorrhagic transformation (**HT**) within 24 hours.[65] HT is a complication of thrombolytic treatment which leads to bleeding in the brain and increased mortality and morbidity.[66] The authors derived a 6-gene profile (SMAD4, INPP5D, VEGI, AREG, MARCH7, and MCFD2) which could differentiate IS subjects who later developed HT from ones who did not. In a validation cohort, 80% sensitivity and 70.2% specificity were achieved.[65] This study showed proof of principle that differences in the peripheral blood transcriptome BEFORE thrombolytic treatment is administered could identify subjects who are at a high risk for developing HT.

Delayed brain injury (DBI) following aneurysmal subarachnoid hemorrhage (aSAH)

DBI complications following SAH, such as delayed cerebral vasospasm (DCV)[67] and delayed cerebral ischemia (DCI)[68] significantly increase mortality and morbidity.[69] However, the pathophysiology of DBI is poorly understood. The search for biomarkers has been reviewed.[70,71] A study by Pulcrano-Nicolas et al[72] performed miRNA-Seq in DCV+ vs DCV− in aSAH patients from the VASOGENE cohort (NCT01779713). It identified elevated miR-3177-3p levels in peripheral blood in DCV+ patients compared

to DCV- patients and proposed miR-3177-3p as a potential candidate biomarker for DCV risk.[72] Pulcrano-Nicolas et al[73] in a separate study in patients from the same VASOGENE cohort analyzed the protein-coding transcriptome and identified a significant difference between the DCV+ and DCV- aSAH groups for S1PR4 gene expression ($\Delta(D_{V3}\text{-}D_0)$). The two time-points were: admission day (D_0) and 3 days before DCV+ patients experienced vasospasm, or the corresponding day for the DCV- aSAH patients (D_{V3}). DCV- patients had lower Δ S1PR4 expression than DCV+ patients. The AUC of the prediction model integrating Δ S1PR4 expression, sex and age was 0.896. S1PR4 and its ligand SP1 are expressed mainly by platelets and play roles in arterial-associated vasoconstriction.[74–76] The authors speculated that following cerebral SAH, platelets release SP1 which triggers vasoconstriction through S1PR4-mediated mechanism.[73] This human data suggests that temporal change of S1PR4 expression is associated with developing vasospasm.

Xu & Stamova et al also investigated the peripheral blood transcriptome of aSAH patients with and without vasospasm with average time post aSAH *ictus* of the blood collections of 10.9 and 8.5 days, respectively.[77] The authors identified differentially expressed genes and differential exon usage between the groups. PCA on the exon expression data separated DCV+ and DCV- patients, suggesting substantial shift in the transcriptome architecture.[77] The significant pathways included cardiovascular signaling pathways such as α-adrenergic, nitric oxide, endothelin-1, renin–angiotensin and thrombin signaling, as well as inflammation and stress response signaling pathways such as CCR3, CXCR4, MIF, fMLP and PPARα/RXRα signaling. The findings were consistent with experimental SAH studies, and a number of these pathways have been investigated in animal models and as potential treatment targets.[77]

Outcome and clinical parameters associated with outcome

Studies identifying the molecular underpinnings associated with outcome following human stroke are of utmost importance as they can help prioritize genes and pathways for follow-up in mechanistic preclinical studies. They can also provide the experimental stroke literature with comparable human data and with the co-expression networks which outcome-associated genes participate in. In addition, as mentioned above, transcriptomic biomarkers of outcome can aid in determining prognosis of individual patients and stratifying patients in clinical trials to improve trial success.

Ischemic stroke outcome

Peripheral blood transcriptome changes associated with severity and outcome following IS have been investigated in several studies. Barr et al[78] identified temporal gene expression changes between day 1 and 2 post *ictus*, and potential biomarkers of 30-day outcome (modified Ranking Score, mRS) (Table 1). The authors found high baseline expression of *TLR2* and *TLR4* associated with poor 30-day outcome. Additionally, *TLR4* expression significantly decreased between day 1 and 2 post *ictus* in patients with good 30-day outcome. The findings suggest *TLR2* and *TLR4* might be potential outcome biomarkers.[78] Amini et al. (described further below) also found *TLR4* expression at 5 h was higher in IS survivors with poor 90-day mRS outcomes (mRS = 3–5) compared to controls, and *TLR2* was a hub gene (a gene with the highest intramodular connectivity, thus potential master regulator in the co-expression module) in a 5 h co-expression module where higher expression was associated with poor 90-day outcome.[79]

Using RNA-Seq, Meller et al[80] investigated the peripheral blood transcriptome of IS with middle cerebral artery occlusion and sex- and age-matched hypertensive controls in African Americans. They stratified the IS subjects into minor (NIHSS 0–5), moderate (6–15), and severe (>15) strokes, and identified 174 exons based on which subjects with different IS severity were separated on PCA and hierarchical clustering. Additionally, they derived a 30-exon panel that predicted the percent improvement between admission and discharge with 100% normalized correct rate (Table 1).[80] The authors studied the transcriptome changes at gene-, exon, and alternatively-spliced transcript level, and suggested exon-level resolution might have the highest biomarker potential.[80]

Raman et al[81] performed peripheral blood transcriptomics from subjects from the INTERSTROKE study[82] on 299 samples in a derivation cohort (25 ICH, 104 IS, 170 controls) and a validation cohort (28 cases, 34 controls). The authors found *MCEMP1* (Mast cell expressed membrane protein 1) was a potential diagnostic biomarker to distinguish between ICH and IS (expressed higher in ICH and IS vs control, and higher in ICH vs IS); and higher *MCEMP1* expression was a prognostic biomarker of worse 30-day mRS outcome (Table 1). MCEMP1 expression decreased over time and thus could potentially estimate time post *ictus*.[81] Additionally, MCEMP1 expression was higher in CE and LVA compared to small vessel IS.[81] MCEMP1 is expressed in mast cells, which are activated following cerebral ischemia and regulate BBB permeability, leading the authors to speculate that

Table 1. Potential predictors of outcome and prognosis in ischemic stroke from clinical studies on peripheral blood RNA.

Outcome	Time of blood draw	Platform	Sample size and outcome definition	Treatment	Level of analyses	Genes	Study
30-day mRS	0–24 h 24–48 h	Illumina HumanRef-8v2 Bead Chips (post-hoc analysis on select TLR Pathway genes)	30-day Good outcome (mRS 0–1), n = 15 30-day Poor outcome (mRS 2–6), n = 8 Validation cohort: Good outcome, n = 4; Poor outcome, n = 4	*Not described*	Gene Level	*TLR2*, *TLR4*	Barr et al., 2015[78]
% NIHSS Improvement	≤24 h	RNA-seq on SOLiD 5500XL	Good prognosis (% NIHSS improvement ≥60%), n = 10 Poor prognosis (%NIHSS improvement <60%), n = 7 Improvement calculated as: (100% X ((NIHSS$_{admission}$-NIHSS$_{discharge}$)/NIHSS$_{admission}$))	11/17 participants received rt-PA	Exon Level (genes presented contain the potential predictor exons)	*PIK3CD*, *VPS13D*, *NRD1*, *NCF2*, *RAB18*, *ANXA11*, *ATF7IP*, *XLOC_027285*, *MON2*, *PPP1R12A*, *PLXNC1*, *C14orf101*, *GOLGA8B*, *CSK*, *NCOR1*, *CRLF3*, *SLC44A2*, *DNM2*, *AKAP8L*, *RBCK1*, *NCOA6*, *GNAS*, *XLOC_089389*, *COPG1*, *PHC3*, *ATP11B*, *FRYL*, *PJA2*, *NCOA2*, *SEMA4D*	Meller et al., 2016[80]
30-day mRS	≤5 days	Illumina HumanRef-8v4 BeadChip TaqMan qPCR	30-day outcome (mRS = 0–2 vs. mRS > 2) 30-day mortality (mRS = 0–5 vs. mRS = 6) Derivation Cohort n = 299 samples: (strokes: 25 ICH, 104 IS, n = 170 controls) Validation Cohort n = 28 cases, n = 34 controls	Some with rt-PA	Gene Level	*MCEMP1*	Raman et al., 2016[81]
90-day mRS	≤3 h, 24h	Affymetrix Human U133 Plus 2.0	90-day Good outcome (mRS 0–2), n = 26 90-day Poor outcome (mRS 3–5), n = 10 Derivation cohort n = 25 Validation cohort n = 11	All participants received rt-PA with or without eptifibatide after the first blood draws	Change in Gene Level Expression Between 24 h and 3 h Δ Expression = 24h Expression − 3h Expression	*AVPR1A*, *CCDC157*, *KCNK1*, *LOC645166*, *MSRB3*, *LINC01541*, *APCDD1*, *RNF150*, *HIPK2*, *LOC100996342*	Amini et al., 2023[79]

MCEMP1 may participate in mast-cell mediated BBB disruption.[81]

Amini et al[79] investigated the acute-phase peripheral blood transcriptome architecture at three time points for each IS subject and associated it with the 90-day mRS outcome. The IS subjects were from the CLEAR trial (NCT00250991)[32] described above. They were stratified based on 90-day mRS outcome (mRS = 0–2, good outcome; mRS = 3–5, poor outcome of survivors). The poor 90-day outcome group revealed many more differentially expressed genes than the good outcome group when compared to VRFCs. Poor outcomes also had significant activation in many inflammatory pathways before and after treatment, such as Acute Phase Response, HMGB1, IL-6, 17 A, STAT3, HIF1α, and NF-κB Signaling pathways. Additionally, poor outcome subjects had suppression in anti-inflammatory pathways such as LXR/RXR and PPARα/RXRα Signaling pathway (Figure 2; for details, please see Supplementary Material). Comparing the poor vs good 90-day outcome group revealed activation of inflammatory and suppression of T-cell specific pathways in the poor vs good outcome groups. The study also found up-regulation of neutrophil-specific, and down-regulation of lymphocyte-specific genes associated with poor 90-day outcome. Moreover, weighted gene co-expression network analysis (WGCNA) revealed modules of co-expressed genes associated with outcome as well as potential master regulator hub genes. A number of the outcome-associated modules contained GWAS signal, including a 24 h-module associated with ordinal 90-day mRS (unpublished data) which was significantly enriched with 15 IS risk GWAS genes,[83] and 6 IS Outcome GWAS genes[84–86] (Figure 3; for details, please see Supplementary Material). These included ADAM23, which has been shown to correlate with early neurological instability, providing evidence for a role of metalloproteinases affecting cell-dell and cell-matrix interactions in influencing outcome.[84] Additionally, Amini et al derived a pilot 10-gene panel of putative biomarkers which predicted poor vs good 90-day mRS outcome with an AUC = 0.88 (Table 1).

Intracerebral hemorrhage outcome

Higher levels of circulating fibrinogen and HMGB1 have been associated with poorer outcomes following human ICH in a review and metanalysis by Kirby et al.[87] Notably, *HMGB1* mRNA level was also found to be up-regulated in peripheral blood of ICH

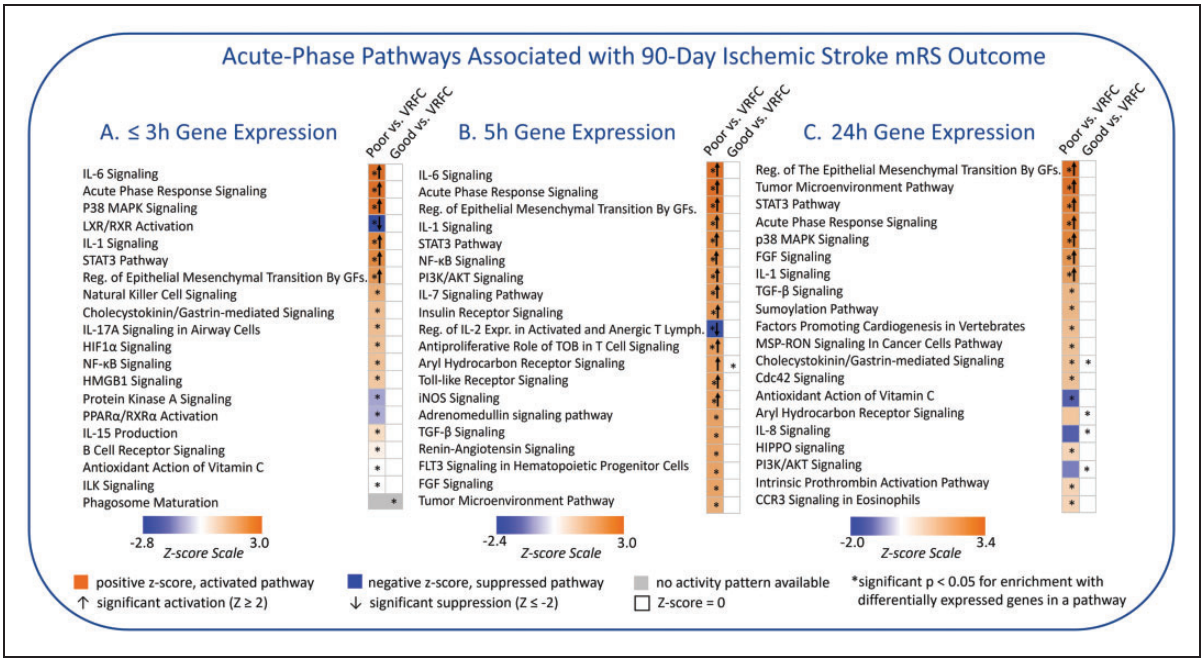

Figure 2. Acute-phase pathways associated with dichotomized 90-day ischemic stroke mRS outcome. Significant pathways enriched in differentially expressed genes between poor 90-day ischemic stroke outcome (mRS = 3–5, survivors) vs. vascular risk factor-matched controls (VRFC), and between good 90-day ischemic stroke outcome (mRS = 0–2) vs. VRFC. The top 20 most significant relevant pathways with activation or suppression are presented for the three time-points after stroke: (a) Gene expression at ≤3 h post *ictus* (before thrombolytic treatment), (b) Gene expression at 5 h post *ictus*, and (c) Gene expression at 24 h post *ictus*. *Reg.* – *regulation*; *GFs.* – growth factors; *Expr.* – expression; *Lymph.* – Lymphocytes. *Modified from Amini et al,* J Neuroinflammation, 2023, PMID: 36691064 published under Creative Commons Attribution 4.0 International License. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/. For details, please see Supplementary Material.

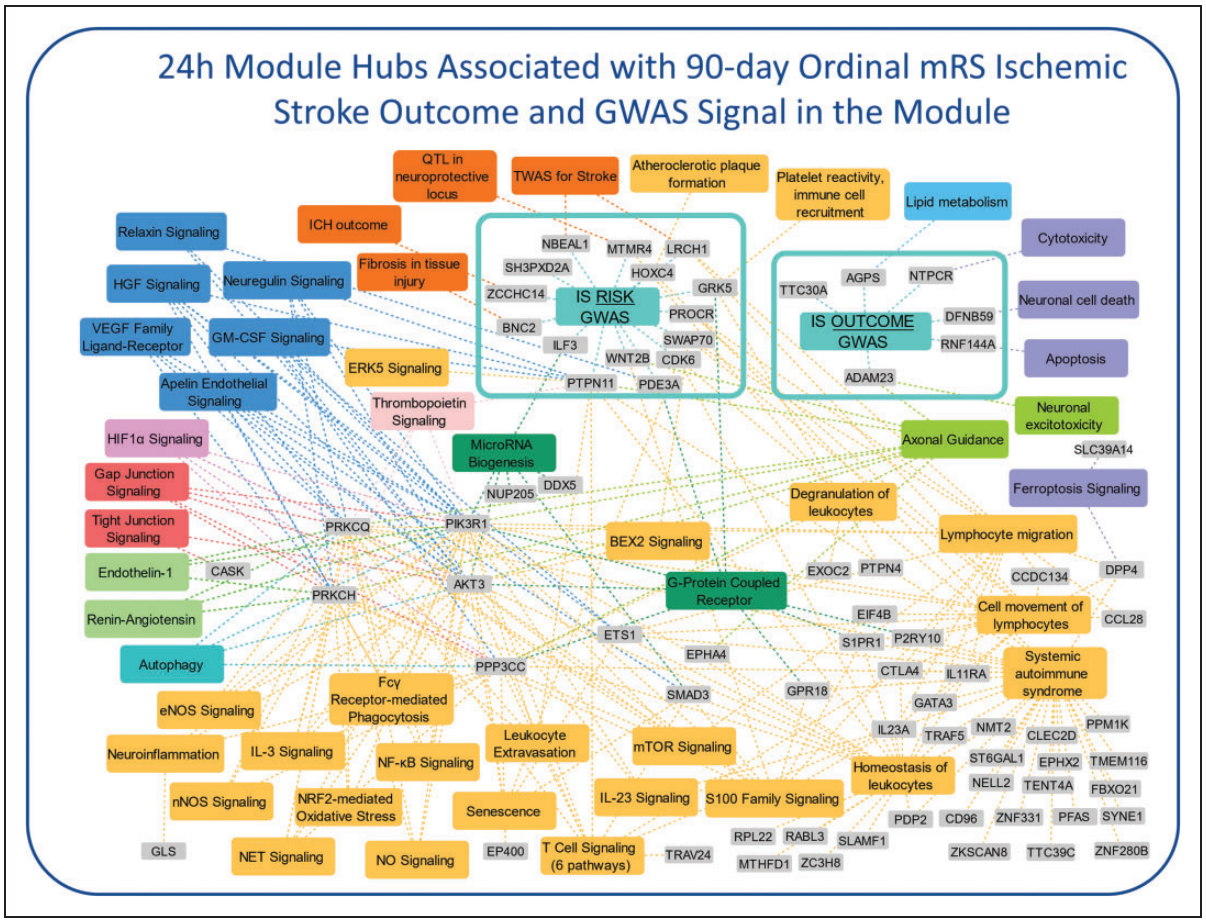

Figure 3. Additional analysis on Amini *et al*, J Neuroinflammation, 2023 (PMID: 36691064). For this review, we associated the gene-co-expression modules we identified in Amini et al, 2023, with the ordinal 90-day mRS ischemic stroke outcome. A 24 h peripheral blood gene co-expression module (24hTurquoise module from Amini et al, 2023) which significantly associated with ordinal 90-day mRS (modified Rankin Score) and with genetic signal in this re-analysis. Displayed are 24 h hub genes, which represent genes with the highest intramodular connectivity, and are thus potential master regulators in this module. Additionally, GWAS signal associated with stroke risk and stroke outcome in the same module are displayed (in the turquoise-colored boxes, module members, not hubs). Each gene is connected to relevant pathways or functions they are involved in based on IPA analysis and literature review. For details, please see Supplementary Material.

patients with larger relative peri-hematoma edema volumes, and HMGB1 Signaling pathway was activated with larger ICH and PHE volumes[88] (see below). A longitudinal transcriptomics study of paired peripheral blood-hematoma effluent samples in living ICH patients provided a unique view of the temporal evolution of ICH. It revealed unique dynamics of the immune response in blood and brain, showing the peripheral blood immune response has a different time course from the one in the brain.[15] The study also associated subacute-phase gene expression with the 365-day mRS outcome (mRS = 0–3, good; mRS = 4–6, poor). The authors found a robust signature of hematoma effluent CD14+ monocytes/macrophages genes associated with 365-day outcome.[15] HIF-1α signaling and glycolysis pathways associated with good patient outcomes.[15] Hematoma effluent or peripheral blood neutrophil genes did not associate with 365-day outcome.[15] *TMEM51* was the only peripheral blood gene expressed in monocytes that associated with 365-day outcome with higher expression in good versus poor outcome. The authors suggest a potentially critical role of hematoma CD14+ monocytes/macrophages in long-term ICH outcome and point out lack of significant association of genes from other cell types to neurological recovery may be due to small sample size.[15]

Association with ICH and perihematomal edema (PHE) volumes

ICH and PHE volumes are key indicators of ICH outcome.[89] Investigating the molecular underpinnings of ICH and PHE volumes could delineate relevant

biological processes and potential treatment targets. Durocher & Knepp et al[88] investigated the ICH peripheral blood transcriptome in the subacute phase to delineate peripheral blood genes and co-expression networks associated with ICH volume, absolute perihematomal edema (**aPHE**) volume, and relative PHE (aPHE/ICH; **rPHE**). The volumetric measures-associated genes and gene co-expression modules were enriched in inflammatory pathways, including NF-κB, TREM1, HMGB1, and Neuroinflammation Signaling, which were most activated with larger ICH and aPHE volumes. Activation of the Leukocyte Extravasation Signaling, the pathway by which peripheral leukocytes infiltrate injured brain, was associated with larger ICH and aPHE volumes. It included adhesion molecules that cause BBB dysfunction, such as MMP9, MMP25, ICAM1, ITGAM, TIMP2, VAV3, and VASP. Additionally, autophagy, apoptosis, HIF-1α, inflammatory and neuroinflammatory response (including TLRs), cell adhesion, platelet activation, T cell receptor signaling, and mRNA splicing were represented in these modules. They were enriched in neutrophil, monocyte, erythroblast, and/or T cell-specific genes. Module hub genes included NCF2, NCF4, STX3, and CSF3R, which are involved in immune response, autophagy, and neutrophil chemotaxis. A module in which reduced gene expression correlated with higher ICH volume and lower rPHE was enriched in T cell-specific genes including hubs LCK and ITK, Src family tyrosine kinases whose modulation improved outcomes and reduced BBB dysfunction following experimental ICH.[88] Many of the findings have been studied in experimental ICH models and have been proposed as potential experimental targets.[37,90] This study provided critical human ICH data for these genes and the networks they potentially modulate. The identified pathways and hub genes may represent novel therapeutic targets.

Aneurysmal SAH (aSAH) outcome

Several studies have identified potential peripheral blood miRNA biomarkers for aSAH outcome (reviewed in[71]). Some of the miRNA biomarkers and the pathways they regulate have also been found to be altered in experimental models of SAH, and thus may be good biomarkers for SAH and perhaps even potential treatment targets (reviewed in[91]). It is possible that composite miRNA-mRNA panels might be even better biomarkers for aSAH outcome.

Biological sex

Sex differences exist for TLR pathways, antigen presenting cells, dendritic cells, macrophages, NK cell and neutrophils from the innate immune system, and in T cells, B cells and immunoglobulins in the adaptive immune system (reviewed in[92,93]). At the peripheral blood transcriptome level, extensive sex differences encompassing the sex-chromosome expressed genes and the autosome expressed genes have been reported.[94] One-third of the female-biased genes are involved in the immune system response.[94] The role of sex in the immune system aging is not well understood. A peripheral blood mononuclear cell study using ATAC-seq, RNA-seq, and flow cytometry in 172 adults revealed sex-specific differences including a greater magnitude of decline in naïve T cell function and B-cell specific loci, and a greater magnitude of increase in monocyte and cytotoxic cell functions in men than in women.[51] Sex differences in the transcriptome architecture increase after age 65, when men have higher innate and pro-inflammatory and lower adaptive activity in corresponding transcriptional pathways.[51] As age is a significant risk factor for stroke, studying sex-differences in the aging immune system in stroke is likely to be very important.

Sex differences have been reported in stroke epidemiology, pathophysiology, treatment and outcome.[95] Efforts are made to address sex differences more adequately.[96] On a molecular level, sex differences in stroke exist in the immune and coagulation systems which have been examined in both preclinical and clinical studies (reviewed in[97–99]). An additional layer of heterogeneity is added by the sex-specific genetic architecture of disease, and for cardiovascular disease-associated traits that are stroke comorbidities and risk factors, such as triglycerides, LDL- and HDL-cholesterol, systolic blood pressure, and lipoprotein (a).[100]

Sex differences in ischemic stroke

There are sex differences in IS incidence, stroke etiology, and outcome, including the fact that males have increased stroke risk in middle age while females tend to have higher stroke risk when older.[101] Females have more cardioembolic stroke.[101] Post-stroke motor disability and mental impairment are higher in older females than older males.[102] Sex differences in treatment efficacy have also been observed.[101] Hormone-dependent and hormone-independent mechanisms are suggested to contribute to sex differences in stroke.[103] Heterogeneity in the peripheral blood IS transcriptome due to sex has been described in the protein coding and non-protein coding regulatory transcriptomes, in IS of different etiologies, and in its temporal evolution.

A much stronger immune response in the protein-coding peripheral blood transcriptome is observed in women with IS compared to men.[104,105] Sex differences were studied in samples from the CLEAR trial

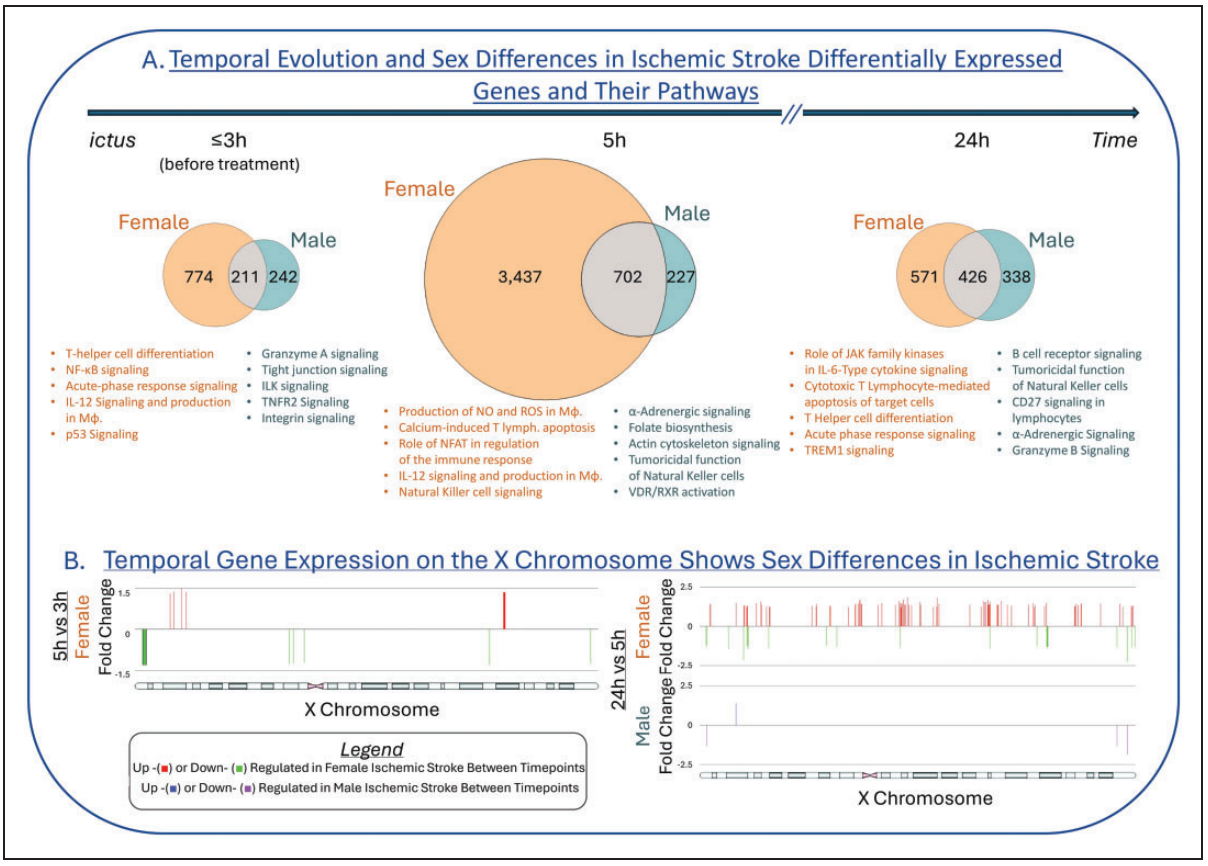

Figure 4. Sex Differences in the Peripheral Blood Transcriptional Response to Ischemic Stroke. (a) Proportional Venn diagrams of the numbers of the differentially expressed genes between ischemic stroke female and control female and ischemic stroke male and control male (Benjamini-Hochberg false discovery rate < 0.05, |fold change| > 1.5) for each time point ($\leq$3 h, before treatment; 5 h and 24 h post *ictus*). The top 5 most significant pathways ($p < 0.05$) in the female-specific and the male-specific response to ischemic stroke at each timepoint are displayed. Modified from Tian & Stamova *et al*, JCBFM, 2012 (PMID: 22167233) with permission. (b) Temporal gene expression of genes on the X chromosome shows sex differences. Gene expression at 5 h vs 3 h of ischemic stroke female vs control female showed 5 genes were up-regulated (red), and 5 genes (7 probe sets) were down-regulated (green) (FDR $p < 0.05$, |fold change|>1.2). The widths of the probe sets are proportional to the Affymetrix Array target region. No genes from the X chromosome were differentially expressed between 3 h and 5 h in ischemic stroke male vs control male. Between 24 h vs 5 h in ischemic stroke female vs control female, there were 98 genes (117 probe sets) up-regulated and 16 down-regulated genes (18 probe sets), while in ischemic stroke male vs control male - 1 gene was up-regulated (blue) and 3 were down-regulated (pink). Modified from Stamova & Tian et al, Stroke, 2012 (PMID: 22052522) with permission. For details, please see Supplementary Material. The Creative Commons license does not apply to the content in Figure 4(b). Use of the material in Figure 4(b) in any format is prohibited without written permission from the publisher, Wolters Kluwer Health, Inc. Please contact permissions@lww.com for further information.

(NCT00250991)[32] with peripheral blood draws at $\leq$3 h (before treatment), 5 h and 24 h post *ictus*[104,105] (Figure 4; for details, please see Supplementary Material). At all three time-points there were more differentially expressed genes in female IS compared to female control than in male IS compared to male control[105] (Figure 4). There were more up-regulated than down-regulated genes in all male- and female-specific gene lists with the exception of 5 h post *ictus* female-specific genes, where the opposite trend was observed.[105] For example, the female-specific genes involved in the calcium-induced T lymphocyte apoptosis process were all down-regulated in IS at 5 h compared to controls and the pathway was predicted suppressed[105] (Figure 5(a); for details, please see Supplementary Material). This may be related to sex differences in T cell subpopulations and function following stroke.[99] Additionally, IS females at 5 h quadrupled the number of differentially expressed genes compared to the pre-treatment 3 h time-point, while IS males only doubled this number (Figure 4(a)). The 5 h timepoint may reflect sex-differences in the response to treatment, as females may have greater benefit from tPA treatment than men.[106] The evolution of the peripheral blood transcriptional response in women and men is presented in Figures 4 (derived from[104,105]) and

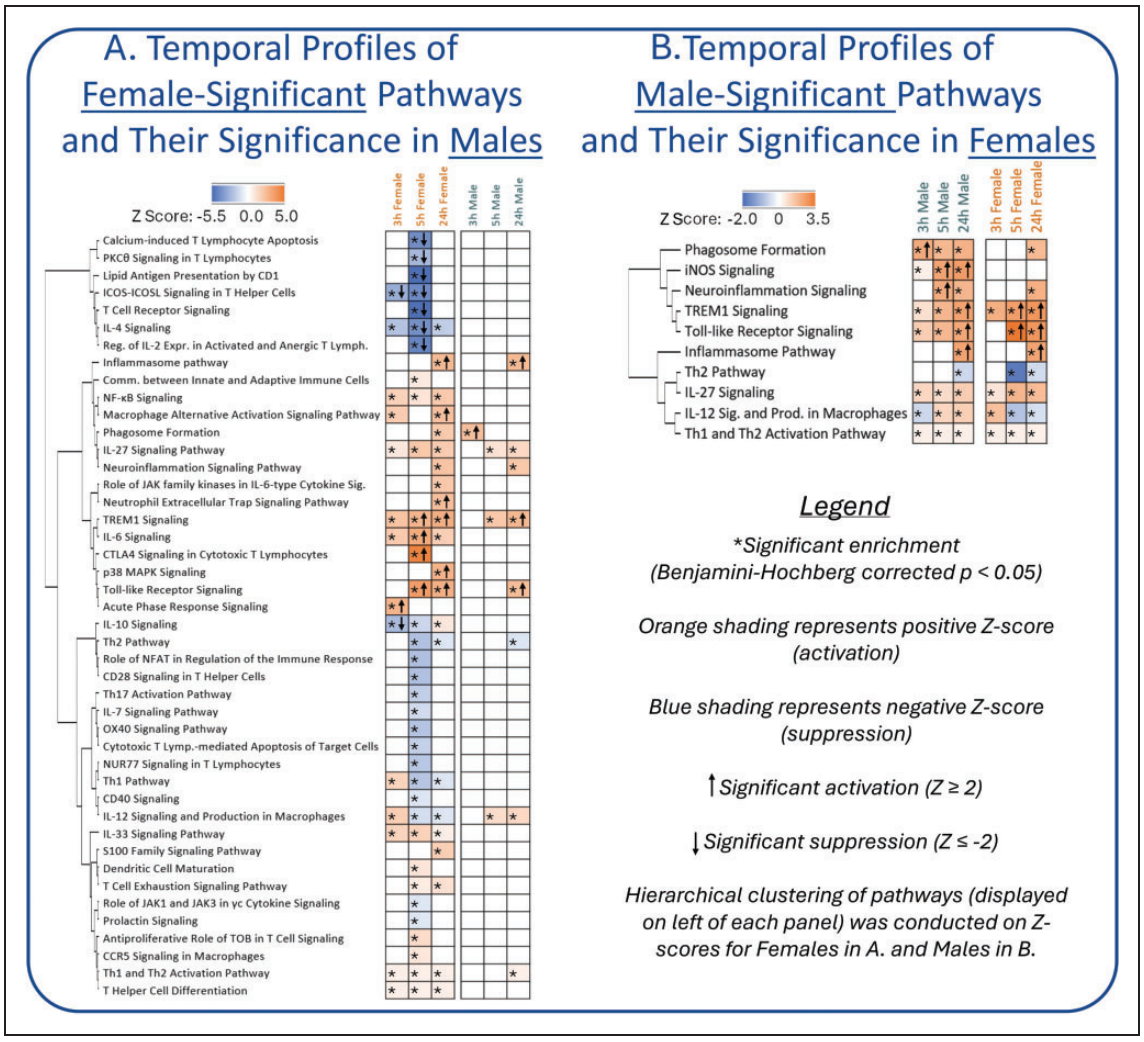

Figure 5. Additional analysis on Tian & Stamova *et al*, JCBFM, 2012 (PMID: 22167233) based on Ingenuity Pathway Analysis of the entire female response – on each of the 985, 4,139, and 997 probe sets that were differentially expressed at $\leq$3 h (before treatment), 5 h and 24 h post *ictus*, respectively between ischemic stroke female vs control female; and on the entire male response – on each of the 453, 929, and 764 probe sets that were differentially expressed at $\leq$3 h (before treatment), 5 h and 24 h post *ictus*, respectively between ischemic stroke male vs control male. (a) Hierarchical clustering of significant Cellular Immunity, Humoral Immunity and Cytokine Signaling Pathways enriched with differentially expressed genes between **female ischemic stroke vs female control**. As a comparison, next to them the significance in male ischemic stroke vs male control in the corresponding pathways is presented. (b) Hierarchical clustering of significant Cellular Immunity, Humoral Immunity and Cytokine Signaling Pathways enriched with differentially expressed genes between **male ischemic stroke vs male control**. As a comparison, next to them the significance in female ischemic stroke vs female control in the corresponding pathways is presented. For both panels, shading is based on the Z score for activation (orange) or suppression (blue) if the pathway is significantly enriched (Benjamini-Hochberg $p < 0.05$; marked with asterisks). Significant activation ($Z \geq 2$) or suppression ($Z \leq -2$) is marked with up and down arrows, respectively. For details, please see Supplementary Material.

Figure 5 (where we performed IPA re-analysis of the complete (sex-specific and common differentially expressed genes combined) female and male responses from[105]; for details, please see Supplementary Material). Women responded with a preponderance of immune/inflammatory molecules and pathways and acute response pathways many of which were predicted activated, such as HMGB1, IL6 and Acute Phase Response (Figure 5(a)). Male responses had smaller number of immune/inflammatory pathways (Figure 5(b)). The female immune response also included genes such as ALOX5, ALOX5P, and CXCL1, which are implicated in the pathogenesis of atherosclerosis by increasing inflammation and causing BBB dysfunction.[107,108] There were also sex differences in cell death pathways, which is consistent with experimental findings (reviewed in[99]). Sex differences in the expression of genes from the X-chromosome showed the same pattern of more genes

and more dynamic changes in IS females compared to males (Figure 4(b)).[104] Similarly, the immune/inflammatory response to cardioembolic stroke was much greater in females at each of the three timepoints and showed temporal differences in the sex-specific granulocyte and monocyte-specific genes.[109]

At the level of the non-coding peripheral blood transcriptome, Dykstra-Aiello et al[110] investigated the sex differences in the long-noncoding RNA (lncRNA) transcriptome in 266 whole-blood RNA samples drawn from IS patients and VRFCs. The subjects were split into derivation and validation cohorts. Notably, in this study there were more lncRNA regulated in IS males than in IS females. Only 6 lncRNA were common between the male and female IS responses, with 4 (mRNAlike lncRNA SPATA3, linc-GUSB-3, linc00671, mRNA-like lncRNA/XIST regulator) expressed in opposite directions. Additionally, IS females had significantly more down regulated lncRNAs over time than IS males. Seven lncRNA with sex-specific differential expression were near vascular risk factor/stroke risk loci. This study also showed sex-specific lncRNA expression changes in each of the three main IS etiologies: CE, LVA and SV IS etiologies.[110] Sex differences in the non-coding regulatory elements of the IS peripheral blood transcriptome are still largely unexplored and warrant further investigation.

Sex differences in ICH

Sex differences in ICH exist in early PHE expansion which is greater in male ICH patients.[111] Male ICH patients also have higher rates of hematoma expansion, 90-day mortality rates,[112] and pneumonia risk.[113] Though females have lower ICH incidence than men, they have a higher 1-year mortality possibly related to older age at ICH onset.[114]

Though little is known about the sex differences in the ICH peripheral blood transcriptome in humans, a small pilot investigation of Deep and Lobar ICH did show transcriptome sex differences in ICH.[44] The study found many more peripheral blood differentially expressed genes in ICH females compared to ICH males.[44] The male ICH response was associated with DNA Methylation and Transcriptional Repression and Apelin Liver Signaling Pathways in deep ICH, and with Protein Binding in Lobar ICH. The female ICH response, on the other hand, was enriched in many immune and coagulation pathways. For example, several T cell pathways were predicted to be significantly suppressed. TLR Signaling and Neuroinflammation Signaling were predicted significantly activated in female ICH, and NF-κB showed a trend towards activation in both lobar and deep female ICH.

The finding of a greater immune response in the coding peripheral blood transcriptome in female ICH compared to male ICH is similar to the sex differences observed in IS discussed above. It is possible males have a greater response to stroke in other layers of the blood transcriptome such as the non-protein coding transcriptome as was observed in IS above.

The human peripheral blood transcriptome findings, the epidemiological sex differences, as well as the experimental stroke literature on sex differences in stroke[97,99] all highlight that it is essential to consider sex in both clinical and pre-clinical studies to improve clinical translation. Understanding sex differences is critical to developing sex-specific medicine in stroke, such as sex-specific treatment targets, prevention strategies, and diagnostic, prognostic, predictive, and pharmacotranscriptomic biomarkers.

Stroke pathophysiology and diagnostic biomarkers

The peripheral blood transcriptome architecture following stroke has been examined in several studies (reviewed in[2,30,50,115]). Since the focus of this review is heterogeneity in stroke subgroups, these studies will not be reviewed here. In addition, diagnostic biomarker development, such as for distinguishing ischemic stroke from ICH and SAH,[116] IS etiology and predicting the stroke mechanism of cryptogenic IS[30] and diagnosing IS[117,118] are discussed in other reviews and not repeated here. However, given the transcriptional heterogeneity documented in this review, it seems likely that separate biomarker panels might be needed to more accurately predict/diagnose molecular subgroups for different sexes with different comorbidities.

Transcriptional heterogeneity in common pathways

Though common pathways have been identified in different phenotypic subgroups, caution must be exercised since different genes may be modulated in the common pathways. For example, even though there were common pathways in the peripheral blood transcriptome of ICH patients with hemorrhage in Deep ICH and Lobar ICH as compared to controls,[44] we found many of the affected genes in the common pathways were ICH-location-specific. For instance, the NF-κB signaling pathway was a common pathway over-represented with differentially expressed genes in lobar ICH and deep ICH. However, the genes were mainly location-specific (Figure 6(a), based on data from[44]; for details, please see Supplementary Material). Genes from the NF-kB pathway are

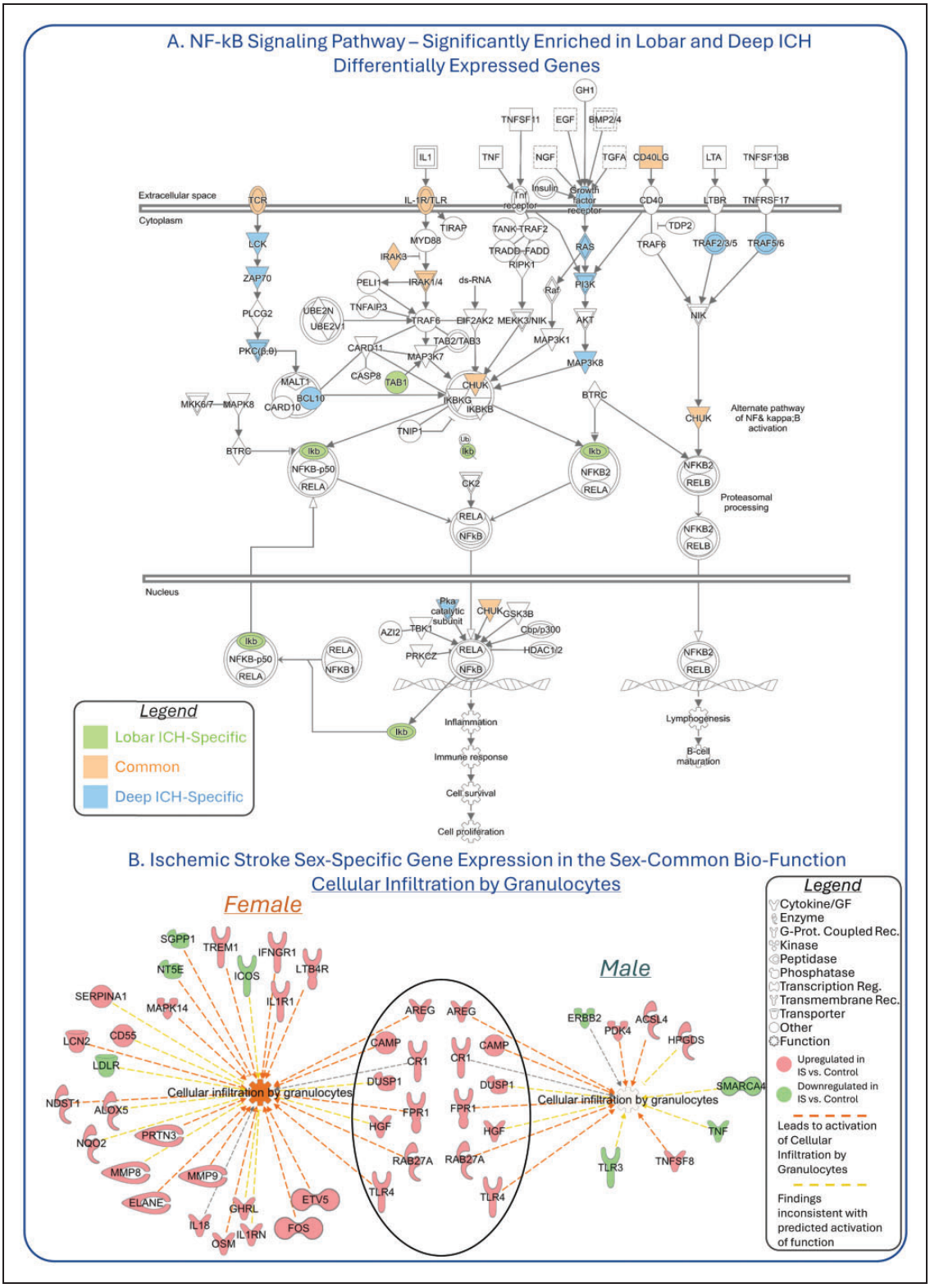

Figure 6. Subgroup-specific heterogeneity in common pathways. (a) Based on data from Knepp et al, Brain Hemorrhages, 2022 (PMID: 36936603). ICH location-specific differentially expressed genes (DEGs) in the ICH location-common NF-κB Signaling Pathway. DEGs between Lobar ICH and Control are highlighted in green; DEGs between Deep ICH and Control are highlighted in blue; DEG in common – in orange. (b) *Based on data from* Tian & Stamova *et al*, JCBFM, 2012 (PMID: 22167233). Sex-specific DEGs in the Cellular Infiltration of Granulocytes Biofunction in female ischemic stroke (IS) vs female control (left) and male IS vs male control (right) at $\leq$3 h post *ictus*, before thrombolytic treatment. Red – genes that were upregulated in IS vs control; green – genes that were downregulated in IS vs Control. The oval circles genes that were common to female and male IS. GF – Growth Factor; Prot. – Protein; Rec. – Receptor; Reg. – Regulator. For details, please see Supplementary Material.

potential treatment targets,[40] but any target should consider ICH location. We also found heterogeneity in the differentially expressed genes in common biofunctions associated with the peripheral immune response to ischemic stroke in males and females. For example, deeper investigation of data from Tian & Stamova et al[105] (described above; for details, please see Supplementary Material), revealed that the Cellular Infiltration of Granulocytes biofunction was significantly over-represented with differentially expressed genes between female IS and female control, as well as between male IS and male control at the $\leq$3 h timepoint (before thrombolytic treatment). However, many of the genes were sex- specific (Figure 6(b); for details, please see Supplementary Material). Out of the 33 genes in females and the 16 genes in males participating in the Cellular Infiltration of Granulocytes biofunction, only 8 overlapped (Figure 6(b)). The largest fraction of the peripheral blood granulocytes are neutrophils. They are among the first cells to infiltrate the injured brain post ischemic stroke. Neutrophils are a major source of matrix metalloproteinases (MMPs) which disrupt the BBB and exacerbate the brain damage.[7] Inflammatory cell infiltration/function has been considered a major therapeutic target in ischemic stroke.[7] Thus, consideration for sex differences in this biological process and/or in its temporal evolution needs to be considered. Overall, molecular heterogeneity in stroke subgroups can have significant repercussions when treatment and prevention strategies are developed and may warrant search for subgroup-specific treatments.

Combinatorial molecular heterogeneity of affected pathways in the human ICH peripheral blood transcriptome: Patient-Specific architecture

As noted throughout this review, significant and pervasive molecular heterogeneity in stroke subgroups exists. To molecularly phenotype this heterogeneity at an even more refined level, we applied an approach we developed previously[119] to delineate the combination of affected pathways at an individual patient-level resolution. To adapt this approach for use in stroke, we reanalyzed the peripheral blood transcriptome of 66 participants (33 ICH, 33 VRFC) we previously utilized to define the inflammatory, regulatory and autophagy gene co-expression modules associated with ICH, and the differentially expressed genes between ICH and VRFC.[11] We originally identified 1,225 differentially expressed genes (FDR $p < 0.05$, $FC > |1.2|$) between the ICH and VRFC transcriptomes.[11] The differentially expressed genes were over-represented in 116 canonical pathways (Benjamini-Hochberg -corrected $p < 0.05$).[11] To evaluate the subject-level heterogeneity here, we first decreased the number of differentially expressed genes by increasing the stringency (FDR $p < 0.05$, $FC > |2|$), which resulted in a total of 105 differentially expressed genes. Ingenuity Pathway Analysis (IPA®) revealed over-representation in 29 significant pathways (Benjamini-Hochberg $p < 0.05$) (Figure 7). Many inflammatory pathways such as Neutrophil Degranulation, Pyroptosis Signaling (an inflammatory cell death pathway), Inflammasome pathway, and IL-6 Signaling were predicted significantly activated or with a trend towards activation in ICH vs VRFC. Some anti-inflammatory pathways, such as IL-10, LXR/RXR signaling, and PPAR/RXR signaling were predicted to be suppressed in ICH vs VRFC.

We then determined which of the 29 pathways were dysregulated/altered in each of the 33 ICH subjects. To do this unique analysis, we performed PCA mapping based on the differentially expressed genes present in each of the 29 significant pathways. In each of these 29 PCAs, we scored an ICH subject as "VRFC-like ICH" if the ICH subject fell within 2 Standard Deviations (SD) of the VRFC centroid, suggesting this ICH subject did not have this particular pathway altered. Alternatively, we scored an ICH subject as "non-VRFC-like ICH" if this ICH subject fell outside the 2SD of the VRFC centroid. PCA of the IL-6 signaling pathway based on the 5 differentially expressed genes in this pathway (CEBPB, IL18RAP, IL1R, IL1RAP, MAPK14) is presented in Figure 7 (for details, please see Supplementary Material), upper left as an example. In this PCA, the blue mesh (VRFC's ellipsoid) is drawn at 2SD of the VRFC centroid. Arrows point to an example of an ICH subject for which the IL-6 signaling pathway is not altered (the ICH subject falls within the blue ellipsoid; VRFC-like ICH) and to an example of an ICH subject for which the IL-6 signaling pathway is altered (the ICH subject falls outside of the VRFC ellipsoid; Non-VRFC-like ICH). We scored each of the 33 ICH subjects as either having a particular pathway not altered, represented by a black rectangle in the hierarchical clustering dendrogram in Figure 7, or as having a particular pathway altered, represented by a gray rectangle in Figure 7. This procedure was performed for each of the 29 significantly over-represented canonical pathways. Hierarchical clustering along the 33 ICH subjects and the 29 canonical pathways' alteration status showed varying combinations of pathways were affected in each of the ICH subjects (Figure 7). Eleven out of the 33 ICH subjects had all 29 pathways altered. The remaining 22 ICH subjects showed different combinations of altered pathways, revealing what we refer to here as combinatorial pathway heterogeneity.

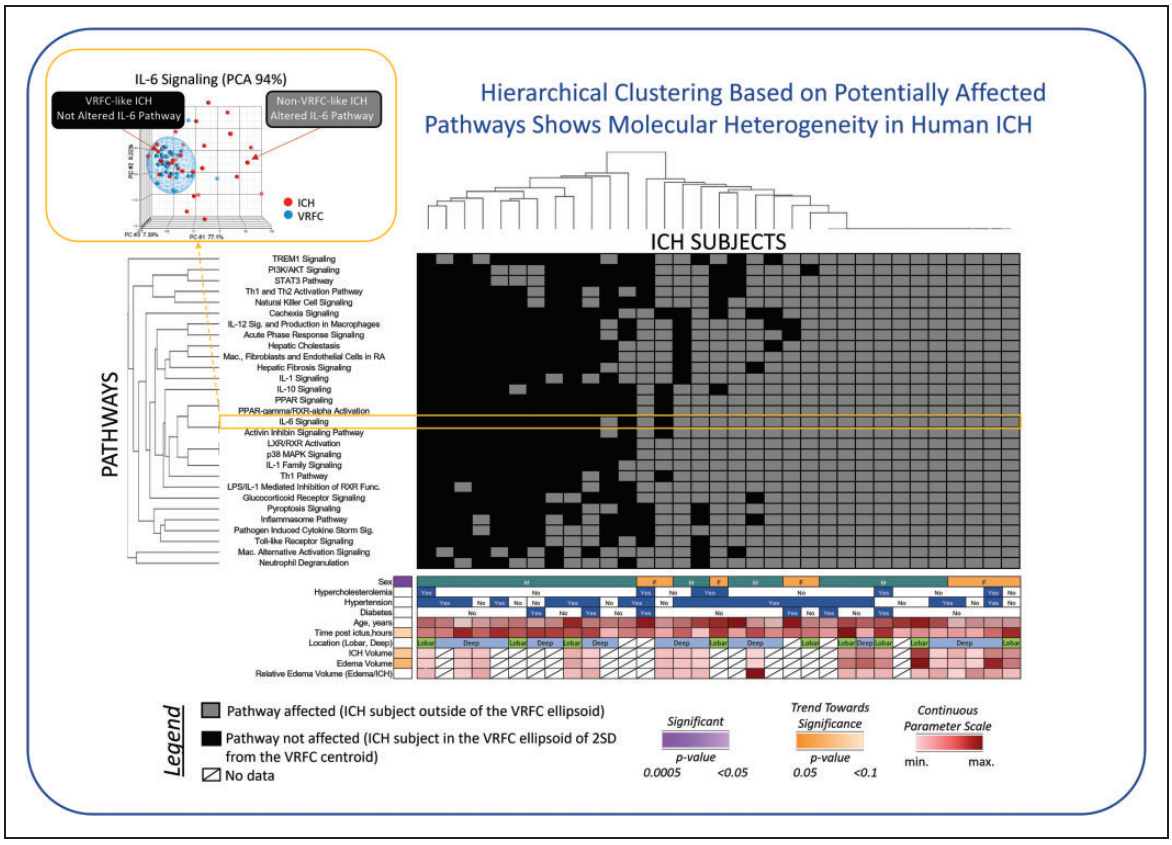

Figure 7. Hierarchical clustering of ICH subjects based on their affected pathways. On the y-axis – pathways significantly enriched with differentially expressed genes in peripheral blood between ICH and vascular risk factor-matched control (VRFC) subjects. On the x-axis – ICH patients. We scored an ICH subject as "VRFC-like ICH" (represented by a black box in the heatmap) if the ICH subject fell within 2 Standard Deviations (SD) of the VRFC centroid, suggesting this ICH subject did not have this particular pathway altered. Alternatively, we scored an ICH subject as "non-VRFC-like ICH" (represented by a grey box in the heatmap) if this ICH subject fell outside the 2SD of the VRFC centroid. PCA of the IL-6 signaling pathway based on the 5 DEGs in this pathway is presented in the upper left as an example. In this PCA, the blue mesh (VRFC's ellipsoid) is drawn at 2SD of the VRFC centroid. Arrows point to an example of an ICH subject for which the IL-6 signaling pathway is not altered (the ICH subject falls within the blue ellipsoid; VRFC-like) and to an example of an ICH subject for which the IL-6 signaling pathway is altered (the ICH subject falls outside of the VRFC ellipsoid; Non-VRFC-like). Clinical and demographic characteristics of each ICH patient are represented on the bottom of the heatmap, as well as the significance of the association between these parameters and the number of altered pathways. For example, Sex was found significant, indicating that the average number of altered pathways in Females was different from the average number of altered pathways in Males. For details, please see Supplementary Material.

Females had significantly more altered pathways than males ($p = 4.8E\text{-}4$). A trend toward significant negative correlation between the number of altered pathways and time post *ictus* was observed ($p = 0.09$). Among the subset of ICH subjects with available ICH- and PHE- volume data ($n = 18$), subjects in which $> 80\%$ of the 29 pathways were altered, had significantly larger ICH and PHE volumes than ICH subjects with smaller number of altered pathways ($p < 0.05$). There was also a trend towards significance for a positive correlation between the ICH and PHE volumes and the number of altered pathways as a continuous variable (Figure 7).

Additionally, we investigated the 29 pathways individually by comparing the ICH subjects that had a particular pathway altered *versus* the subjects that did not have this pathway altered and associated them with different clinical and demographic parameters (Figure 8; for details, please see Supplementary Material). Overall, of the pathways which had a trend towards significance ($0.05 \leq p < 0.1$, orange color scale) or were truly significant ($p < 0.05$, purple color scale), there were more females with the particular pathway altered, the ICH patients were older, the time post *ictus* was shorter, and/or the ICH and PHE volumes were larger.

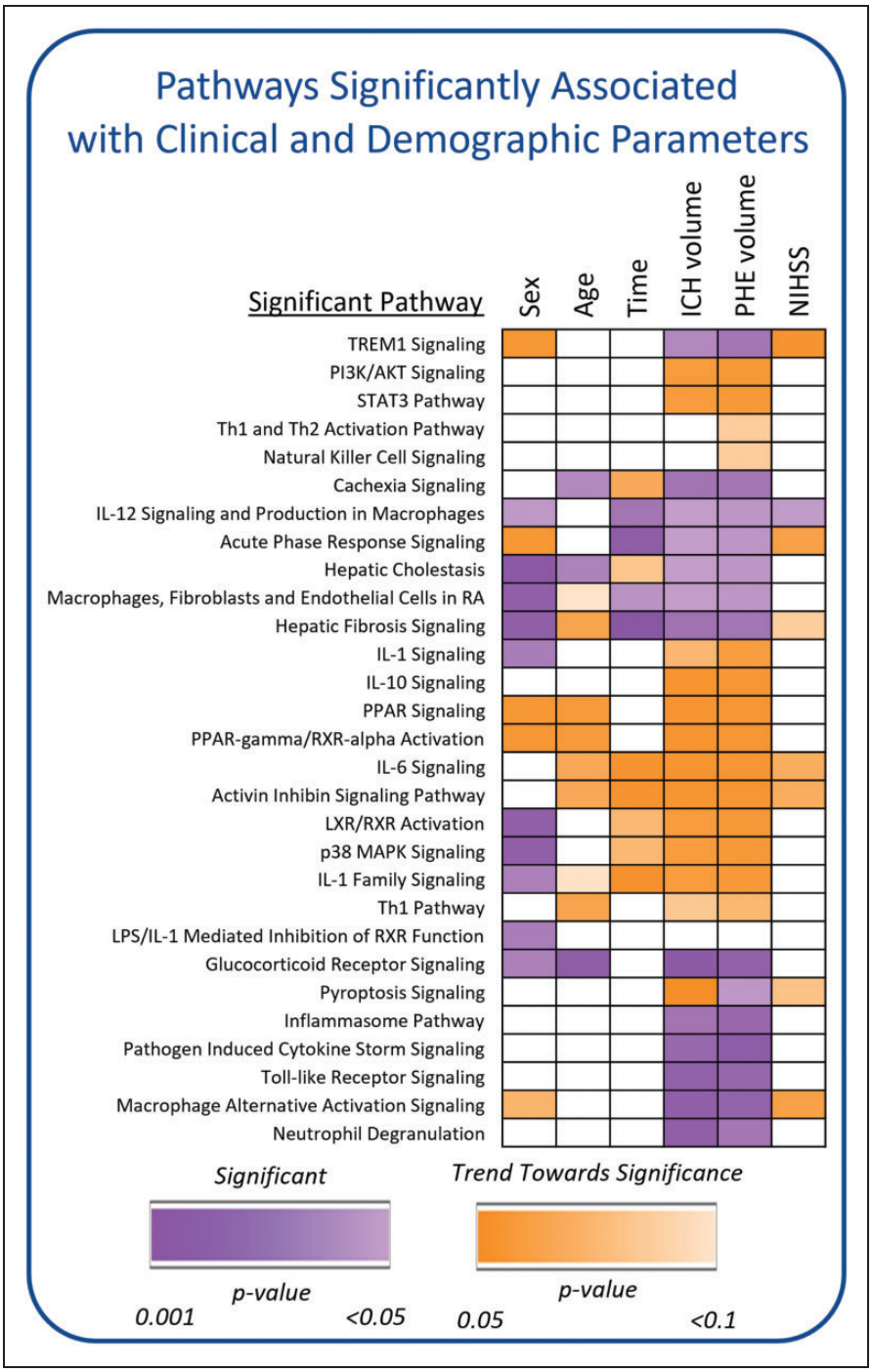

Figure 8. Association between significant pathways from Figure 7 and clinical and demographic parameters. The heatmap represents differences between the ICH subjects with a particular pathway altered (non-VRFC-like ICH) *versus* the ICH subjects in which the pathway is not altered (VRFC-like ICH). For example, ICH subjects with altered TREM1 signaling had higher ICH volume compared to those with not altered TREM1 Signaling. See Figure 7 legend for details. Significant differences ($p < 0.05$) are presented in a purple scale; a trend towards significance ($0.05 \leq p < 0.1$) – in an orange scale; non-significant differences ($p \geq 0.1$) – in white. For details, please see Supplementary Material.

We propose that this method can identify the combinatorial heterogeneity in ICH altered pathways, based on ICH-patient level pathway subgroups. The findings further our understanding of the ICH molecular heterogeneity at an individual-patient level. The proposed approach can be applied to other phenotypic levels, such as proteome, metabolome, epigenome, and imaging data. It strongly underscores

potential repercussions for choosing treatment targets and may provide consideration in identifying candidate patients in clinical trials targeting a particular pathway. For example, 64% of the subjects (21 out of the 33 ICH subjects) in Figure 7 showed potential alterations in the PPARγ/RXRα Activation pathway. PPARγ agonists have been shown to improve experimental ICH outcomes.[120] Thus, identifying which ICH subjects have alterations in this pathway may help pinpoint potential candidates in clinical trials targeting this pathway. The approach may contribute to the overarching goal of advancement towards precision medicine in stroke. Combining this approach with recently developed framework for automatically identifying responsive subgroups regarding treatment effects from randomized clinical trial data[121] may further help advance personalized stroke treatments.

Conclusions

The peripheral immune and coagulation responses contribute to injury and repair mechanisms in the injured brain following stroke. This is underlined by molecular changes at many biological levels including the transcriptome. Transcriptomic studies in peripheral blood following human stroke have revealed considerable heterogeneity in the transcriptome architecture in subgroups related to etiology, comorbidities, biological sex, outcome, response to treatment, temporal evolution, cell-specific responses, and even common pathways. Additionally, we show that subject-level combinatorial heterogeneity exists with different stroke subjects having different combinations of pathways potentially altered. Differences in the individual's response to stroke reveal molecular subgroups and emphasize the need for precision medicine in future treatments and therapeutics. Knowledge of the peripheral transcriptome architecture in human stroke is evolving, and new technologies, such as single-cell RNA sequencing (reviewed by Shi, Chen, & Zhang et al in this special issue) and long-read RNA sequencing (for directly examining the transcript-level resolution) will continue refining it. Additional studies deciphering deeper differences in the human stroke peripheral blood transcriptome, such as ones due to RNA modifications and RNA editing in stroke subgroups, as well as long-distance communication between the brain and peripheral blood via extracellular vesicles, will help decipher stroke phenotypic subgroups. The molecular heterogeneity may have significant repercussions for the development of treatment targets and prevention strategies, and sub-group specific approaches may be needed to advance towards personalized medicine.

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Supplementary material

Supplemental material for this article is available online.

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