SAFE-T Consortium

SAFE-T Consortium Work-Package n°4 Drug-induced Vascular Injury (DIVI)

in collaboration with the Predictive Safety Testing Consortium (PSTC) Vascular Injury Working Group (VIWG)

Summary Data Package supporting Clinical Biomarkers of Drug-Induced Vascular Injury

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List of Abbreviations

ACTA2	Smooth muscle alpha actin
ANCA	Anti-Neutrophil Cytoplasmic Antibody
ANGPT2	Angiopoietin 2
AUC/AUROC	Area under Receiver Operating Characteristic Curve
BQRT	Biomarker Qualification Review Team
CALD1	H-caldesmon
CMA	Classification for MicroArrays
CNN1	H1-calponin
CRO	Clinical Research Organization
CRP	C-reactive protein
CDISC	Clinical Data Interchange Standards Consortium
CDIV	Clinical Drug-induced vasculitis
D/N	Degeneration, necrosis, apoptosis
DILI	Drug-induced liver injury
DIKI	Drug-induced kidney injury
DIVI	Drug-induced vascular injury
EC	Endothelial cell
eCRF	Electronic case report form
ELISA	Enzyme-linked immunosorbent assay
EMA	European Medicines Agency
ESM-1	Endocan
EU	European Union
FDA	Food and Drug Administration
H/H	Hypertrophy, hyperplasia
Hr	Hour(s)
ICAM1	Intercellular adhesion molecule 1
IL	Interleukin
IMI-JU	Innovative Medicines Initiative-Joint Undertaking
IP10	Interferon gamma induced protein 10
IPRG	Interdisciplinary Pharmacogenomics Review Group
LoS	Letter of Support
MCP-1	Monocyte chemotactic protein 1
MIG	Monokine induced by gamma interferon
MIP-1α	Macrophage inflammatory protein 1 alpha
MIP-3β	Macrophage inflammatory protein 3 beta
MSD	Meso-scale Discovery
NBF	Neutral Buffered Formalin
NO	Nitric oxide
PAN	Polyarteritis nodosa
PGWP	Pharmacogenomics Working Party

PSTC	Predictive Safety Testing Consortium
LLOQ	Lower limit of quantification
ROC	Receiver Operating Characteristic
SAFE-T	Safer and Faster Evidence-based Translation
SAWP	Scientific Advice Working Party
SDF-1	Stromal cell derived factor 1
SDTM	Study Data Tabulation Model
SELE	E-selectin
SM	Smooth muscle
SMTN	Smoothelin
SOPs	Standard Operating Procedures
TAK	Takayasu's Arteritis
VCAM1	Vascular cell adhesion molecule 1
VEGFA	Vascular endothelial growth factor A
TAGLN	Transgelin
TIMP1	Tissue inhibitor of metalloproteinase 1
TNF	Tumor necrosis factor
TSP-1	Thrombospondin 1
VI	Vascular Injury
VIWG	Vascular Injury Working Group
VIS	Variable Importance Score
VSMC	Vascular smooth muscle cell
WP	Work Package

1 Executive Summary

A Letter of Support (LoS) was issued by FDA on November 7, 2016, and by EMA on November 7, 2017, to the Safer and Faster Evidence-based Translation (SAFE-T) Consortium and the Predictive Safety Testing Consortium (PSTC) to encourage the further study and use of soluble biomarkers of endothelial cell injury and inflammation, and when available, soluble markers of vascular smooth muscle cell injury, as exploratory diagnostic markers for drug-induced vascular injury (DIVI) in early clinical drug development. The LoS can be found at the following links: FDA DIVI LoS and EMA DIVI LoS. The purpose of this document is to provide supportive information for the candidate DIVI biomarkers listed in the published LoS in order to encourage the conduct of nonclinical and exploratory clinical analyses to evaluate the translational relevance of changes in these candidate DIVI biomarkers. This information includes biological rationale for the candidate biomarker selection, both preclinical and clinical studies completed and planned future studies to support biomarker qualification.

Although not qualified by FDA or EMA, these translatable biomarkers, alone or in panel(s) (herein referred to as "biomarkers"), are anticipated to reflect DIVI affecting vascular smooth muscle cells and endothelial cells, as well as the associated inflammatory response, as determined by histopathologic endpoints in rodents. It is envisioned that the vascular injury biomarkers will ultimately be used in healthy volunteers with no concurrent vascular disease to monitor for vascular safety in early clinical trials. These biomarkers will be used when such injury has been demonstrated to be monitorable by translatable biomarkers in animal studies of similar duration with the same test agent (including small and large molecule therapeutics). Applying the biomarkers in initial single and multiple ascending dose clinical studies could help inform planned dose escalations or continued dosing schedules.

The biomarker candidates were selected based on their association with the three main histopathologic features involved in preclinical DIVI: damage to vascular endothelium, damage to smooth muscle, and inflammation. Because one or more of these features is typically involved in preclinical DIVI and required to confer specificity to the vasculature, it is likely that combinations, or panels, of multiple biomarkers will be required for clinical use. Candidate biomarkers for use in clinical studies could include one or more of the following: endothelial cell proteins (E-Selectin, P-Selectin, sICAM-1, sVCAM-1, thrombomodulin, TIMP-1, and VEGF); smooth muscle cell proteins (calponin, caldesmon); and inflammatory factors (CRP, GROa, NGAL, sICAM-3, IL-6, IL-8, IP-10, I-TAC, MCP-1, MIG, SAA, MIP-1, TNFRSF1A). The literature evidence is summarized in Section 4.5 where the rationale for identifying each of the biomarkers as "candidates" is outlined, along with references to support their inclusion in the qualification process. Experimental data supporting the non-clinical and clinical biomarkers are included in Section 5.2 and Section 6.2, respectively.

A consistent interpretation of the clinical results is that combinations of vascular injury biomarkers can discriminate healthy volunteers from patients with disease and with better performance than individual biomarkers. For the clinical markers that are being analyzed using a multivariate Random Forest model, there are several statistical approaches that can be used to rank the markers according to their "importance" or contribution to the model. However, there are several factors that make it premature to further narrow the biomarker list based on the current data set. First, as recently noted by the Biomarker Qualification Review Team (BQRT), the analytical validation performed to date has revealed "broad precision

profiles" of the biomarker assays that limit our confidence in the ability to generate comparable results in repeated measurements with the same platform. Also, as discussed by the BQRT, we are reporting "exploratory observations obtained only from a small sample subset" of our overall clinical study; therefore, more subjects are needed to confirm these responses once the analytical validation issue has been addressed. And finally, it is not yet clear how best to combine the different biomarker profiles from our two clinical models of vascular injury (vasculitides patients with an acute flare on chronic injury, and balloon angioplasty patients with a known onset of acute injury) into a unified predictive model of DIVI.

Therefore, we encourage the conduct of nonclinical and exploratory clinical analyses to evaluate the translational relevance of changes in the expression of candidate DIVI biomarkers reported in this document. Moreover, data sharing and integrating data across trials can foster an accelerated path for numerous drug development programs. In light of the circumstances outlined above, the data we have generated remain promising, and the issued FDA and EMA LoS provide a useful mechanism to encourage others in the field to address gaps and facilitate the ultimate goal of clinical biomarker qualification.

2 Introduction

2.1 Overview of SAFE-T and PSTC

The Safer and Faster Evidence-based Translation (SAFE-T) Consortium is a non-profit, public-private partnership that started its work in June 2009 under the European Union (EU) Innovative Medicines Initiative-Joint Undertaking (IMI-JU). The objective of the IMI-JU is to support projects for the development of tools and methodologies to address key "bottlenecks" in the pharmaceutical research and development process, similar to the US Food and Drug Administration (FDA)'s Critical Path Initiative. The overall objective of the IMI SAFE-T consortium is the regulatory qualification of clinical safety biomarkers of drug-induced injury to three organs; kidney (DIKI), liver (DILI) and vasculature (DIVI) in humans using peripheral samples such as blood and urine (Matheis et al., 2011).

The Predictive Safety Testing Consortium (PSTC) was formed in 2006, and brings together pharmaceutical companies to share and validate innovative safety testing methods under advisement of the FDA and the European Medicines Agency (EMA), and submit them for formal regulatory qualification when appropriate. The SAFE-T consortium has collaborated from the beginning with the PSTC based on shared objectives, and in 2014, a legal agreement, which formalized the collaborative efforts, was signed. The collaboration between PSTC and SAFE-T addressed, among other things, the selection of biomarkers and setting normal ranges for new biomarkers as defined in healthy volunteers.

The IMI SAFE-T project was finalized in June 2015. Indeed, based on the results presented in this document, FDA on November 7, 2016, and EMA on November 7, 2017, issued a LOS.

2.2 Drug-Induced Vascular Injury Work Package 4 Objectives

The DIVI work package 4 (WP4) of the SAFE-T Consortium and vascular injury working group (VIWG) of the PSTC aim to address the current lack of sensitive and specific clinical

tests to diagnose, predict and monitor drug-induced injury to blood vessels, which is a major safety hurdle in drug development.

The primary objective was to qualify translational markers to detect and/or monitor the potential occurrence of DIVI in clinical studies.

Secondary objectives associated with this primary goal were:

- To gain regulatory acceptance for use of those markers in defined clinical contexts.
- To characterize the correlation of the biomarkers with the severity of vascular injury, and the correlation with disease progression
- To determine the between- and within- subject variability of the soluble biomarkers in healthy individuals, establish the normative ranges and identify the factors that influence these biomarkers
- To characterize the effect of common diseases (e.g., diabetes, atherosclerosis) on the specificity of the biomarkers
- To perform mechanistic understanding studies to support the qualification of DIVI biomarkers

3 Proposed Context of Use

3.1 Context of Use Statement

The proposed Context of Use statement is as follows:

The panel of vascular injury safety biomarkers may be used in conjunction with the totality of information (concurrent standard circulating or functional biomarkers, on/off target and pathophysiologic mechanism of toxicity, safety margin, indication, etc.) in healthy volunteers with no concurrent vascular disease to monitor for vascular safety in early clinical trials. These biomarkers will be used when such injury has been shown to be monitorable by translatable biomarkers in animal studies of similar duration with the same test agent. Applying the biomarkers in initial single and multiple ascending dose clinical studies could help inform planned dose escalations or continued dosing schedules.

3.2 Intended Use

3.2.1 Organ injury of interest

The biomarkers are intended to detect vascular injury as identified in preclinical toxicology studies.

3.2.2 Intended use populations

The biomarkers are intended for use in healthy volunteers only. Studies required to determine specificity of biomarkers in patients with inflammatory or non-inflammatory diseases have not been completed.

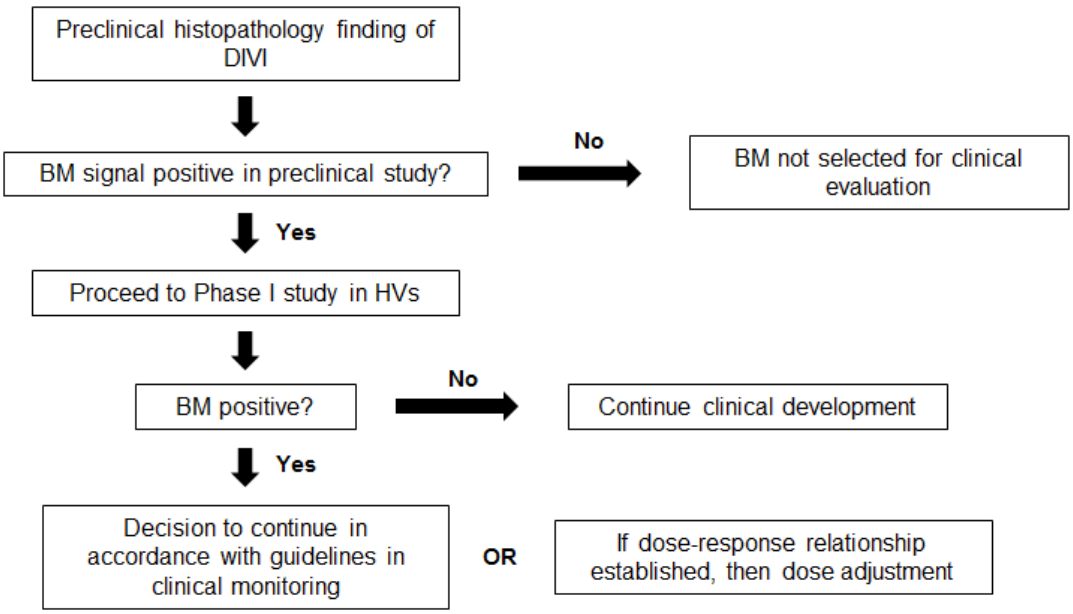
3.2.3 Proposed stages of drug development for the use of selected biomarkers

Because the biomarkers are intended for use in healthy volunteers, their use would be restricted to Phase 1 studies only.

3.2.4 Decision tree for use of biomarkers in drug development

Figure 3-1 shows a proposed schematic that outlines use of the DIVI biomarkers in drug development.

Figure 3-1 Proposed schematic that outlines use of the DIVI biomarkers in drug development



3.3 Description of biomarker assay

The proposed biomarker assay will be a panel of soluble proteins measured in serum from healthy volunteers. It is anticipated that a multivariate classification model will be constructed from a subset of the markers outlined in Section 4.5.2.

4 Background and History

4.1 Overview of specific organ injury

Preclinical DIVI is a phenomenon encompassing a group of histopathologic observations seen in the rat, dog, monkey, and pig (Kerns et al., 2005) that share certain pathologic characteristics but are thought to be pathogenically diverse. Vascular lesions, primarily arterial, can be induced within hours of drug administration; affected animals may show no

clinical signs, and in some cases these lesions might be reversible. While reported and postulated mechanisms are varied, vascular injury in animals is thought to be induced by either altered hemodynamic forces (shear and/or hoop stress), direct drug-induced toxicity, and/or immune-mediated injury of the endothelium and/or medial smooth muscle. Currently, histopathology is the only reliable method of diagnosis for this condition, although promising soluble candidate biomarkers mechanistically linked to the different tissues involved in DIVI have been identified (Brott et al., 2005). For a recent review, see Mikaelian et al. (2014).

4.2 Use and limitations of current tools

Most early reports in the literature described DIVI caused by systemically vasoactive compounds (i.e., those that cause changes in heart rate and systemic blood pressure) but more recently DIVI has been reported/found with compounds that do not alter systemic hemodynamics but may do so in local vascular beds (Louden et al. 2000). While heart rate and blood pressure can be used as biomarkers of vascular injury for those compounds causing systemic hemodynamic changes, this is not true for compounds with only localized vasoactivity or with a different mode of action. The absence of noninvasive biomarkers that can be used to monitor potential vascular injury in humans has increased drug attrition and thus had a profound impact on drug development. In addition, histological evidence of DIVI in the preclinical studies with low or negative safety margins has delayed clinical development of these compounds. For these reason, there has been a search for circulating biomarkers that can detect the onset, progression, and reversibility of DIVI.

4.3 Absence of reference standard/gold standard

In absence of a biomarker gold standard, we propose using the current diagnostic criteria from multiple surrogate populations with human vascular diseases as the basis for determining biomarker performance in exploratory and confirmatory studies. The performance of a biomarker versus the diagnostic criteria of an array of vascular diseases will give insight on the vascular specificity and mechanistic profile of the biomarker(s).

4.4 Preclinical – clinical translation strategy

The similarities in vascular morphology among species provide a basis for the detection of biomarker profiles that reflect the pathophysiologic status of the various vascular compartments. While the extent of involvement of the vascular compartments and their location may vary between human idiopathic vasculitis and preclinical injury, the similarly affected vascular compartment provides a potential source of vascular related biomarkers. Biomarkers are intended to be qualified against three primary features of histopathologic change in humans and animals: endothelial change, smooth muscle damage and inflammation. It is hypothesized that similar histopathology between preclinical DIVI and vascular injury/disease in humans will lead to overlapping biomarker signatures. Therefore, histopathologic changes were identified as the link between the preclinical and clinical conditions, and as a logical anchor against which to investigate vascular injury (VI) biomarkers for qualification in DIVI caused by various mechanisms.

The disease populations for biomarker qualification were selected to cover the histopathological features found in DIVI as previously described. The selected patient populations include diseases involving vessels of different types and sizes, different vascular

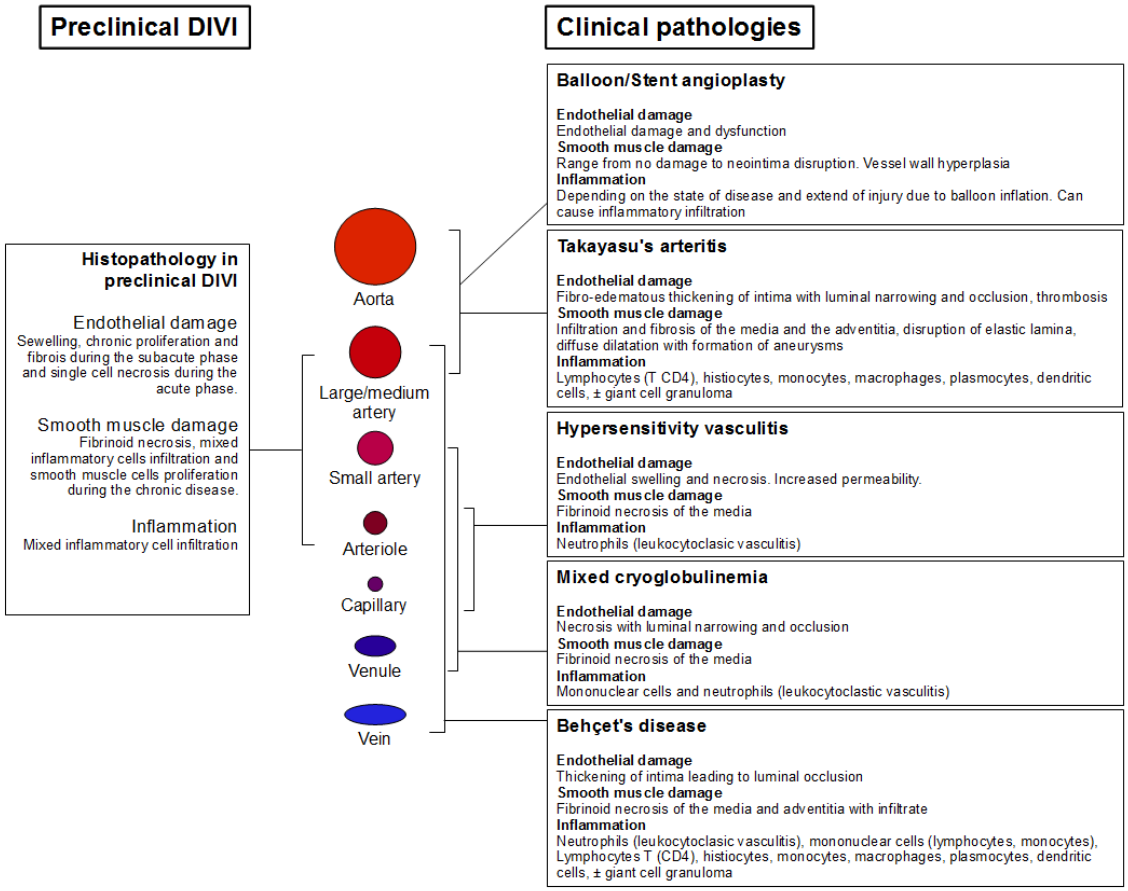
compartments, and various types of infiltrating inflammatory cells. Patients with clinical drug-induced vasculitis (CDIV) would have been preferred for qualification studies but we concluded that the limited access to this population made it impractical to include. Instead, we chose patients presenting with large-size vessel vasculitides (Takayasu's arteritis, Behçet's disease), medium-size vascular disease (patients undergoing balloon/stent angioplasty; see below), or small-size vessel (arterioles, capillaries and venules) vasculitides (hypersensitivity vasculitis, mixed cryoglobulinemia). Such selection criteria also permitted us to include diseases affecting different parts of the artery wall, such as the endothelium (hypersensitivity vasculitis, mixed cryoglobulinemia, Behçet's disease), the media (patients undergoing balloon/stent angioplasty) or the adventitia (Takayasu's arteritis). It also covered different types of vessels such as arteries (Takayasu's arteritis, patients undergoing balloon/stent angioplasty) and veins (Behçet's disease). For the vasculitis patients, inclusion criteria were based on "standard" definitions of disease as recently described by Jennette et al. (2013).

When possible, biomarkers were assessed in the same patient, at different time points i.e. during remission (non-active phase) and during a relapse (active phase). Patients in active phase generally present acute histopathologic alterations of the vessel, whereas vessels from patients in the non-active phase do not exhibit these injuries. Relapse is usually diagnosed based on a combination of clinical, biological, and sometimes imaging tests. Comparisons between patients in active phase to those in remission will help determine which types biomarkers are meaningfully differentiated during the active phase (i.e. to reflect acute injury). Patients in non-active phase should make a useful control group for the specific association of a biomarker signal with vascular histologic damage, rather than with inflammation. Histologic characteristics of the various patient populations are summarized in Table 4-1. Figure 4-1 shows the overlap between the histopathologic features/tissue distribution of DIVI findings and the clinical vascular disorders, as the basis of selecting our patient populations.

Table 4-1 Summary of histologic changes in various human vascular conditions

Pathology	Location	Endothelial damage	Smooth Muscle damage	Inflammatory cells
Clinical Drug-induced vasculitis (CDIV)	Usually affects small and medium-sized vessels; Generally affects the skin, though sometimes kidney, lung, and other organs	Endothelial cells swelling Immune complex deposition	Fibrinoid necrosis	Mixed inflammatory cell infiltration
Takayasu's Arteritis (TAK)	Large ± medium vessel vasculitis; Aorta and proximal branch arteries	Fibro-edematous thickening of intima with luminal narrowing and occlusion, thrombosis	Infiltration and severe fibrosis of the media and adventitia, disruption of elastic lamina, diffuse dilatation with formation of aneurysms	Lymphocytes (T CD4), histiocytes, monocytes, macrophages, plasmocytes, dendritic cells, ± giant cell granuloma
Behcet's Disease	Large, medium and small vessel vasculitis; Arteries and veins (e.g., pulmonary, cerebral)	Thickening of intima with luminal narrowing and occlusion, thrombosis	Infiltration and fibrinoid necrosis of the media and adventitia, aneurysms	Neutrophils (leukocytoclastic vasculitis), mononuclear cells (lymphocytes, monocytes), plasma cells
Hypersensitivity Vasculitis	Small ± medium vessel vasculitis; Arterioles, capillaries, venules	Endothelial swelling and necrosis, increased permeability	± Fibrinoid necrosis of the media	Neutrophils (leukocytoclastic vasculitis)
Mixed cryoglobulinemia	Small ± medium (in case of PAN-like vasculitis) vessel vasculitis Arterioles, capillaries, venules	± Necrotizing vasculitis with fibrinoid necrosis, luminal narrowing and occlusion by thrombosis (in case of PAN-like vasculitis)	± Fibrinoid necrosis of the media (in case of PAN-like vasculitis)	Mononuclear cells (lymphocytes, mononyctes) ± leukocytoclastic vasculitis
Balloon angioplasty/stent	Coronary arteries (large/medium)	Angioplasty /stent can cause endothelial damage/dysfunction and maybe endothelial inflammation	Depending on extent of inflation, angioplasty effects range from no damage to arterial neointimal disruption - inflammatory infiltration and thickening of vessel.	Depending on state of the disease and injury with angioplasty/stent

Figure 4-1 **Diagrammatic representation of overlapping blood vessel involvement and histopathologic manifestation in preclinical DIVI and surrogate populations.**



The selection of patients undergoing balloon angioplasty was based on the goal of including a population with acute injury to the vessel wall and endothelium. Following a screening visit, a sample of blood was to be collected to establish a baseline biomarker level; patients were then to undergo balloon angioplasty and further blood collections were to be performed within 24 hours of, and three months after, the procedure. Several studies have shown the activation of gene expression and release of biomarkers within 24 hours of angioplasty (Bonello et al. 2006; Sardella et al. 2006). For example, Interleukin (IL)-1β and IL-6 levels were significantly increased in the coronary sinus of patients 20 min after angioplasty (Sardella et al. 2006). An additional observational study in patients undergoing angiography was performed to determine the association of biomarkers with atherosclerosis. Patients were to undergo angiographic examination and assessment of arterial stenosis, along with determination of biomarker range.

Anti-Neutrophil Cytoplasmic Antibody (ANCA)-associated vasculitis (Wegener's granulomatosis and microscopic polyangiitis) was not a focus of the project because of a large study already published on promising biomarkers in these diseases (Monach et al. 2013;

Monach et al. 2011). Other pathologies were excluded based on the challenges of clinical studies (i.e., rare pediatric diseases such as Kawasaki's disease and Henoch-Schonlein purpura). Also, despite its many similarities to CDIV, hypersensitivity vasculitis was also not included in the initial qualification studies because, like CDIV, this disease is also rare and blood samples are hard to obtain.

4.5 Biological rationale for each candidate biomarker selection (including literature review)

4.5.1 Preclinical Biomarkers

A list of potential biomarkers, including those for endothelial activation/damage (Table 4-2), vascular smooth muscle damage (Table 4-3), and inflammation (Table 4-4), was collated from the literature, internal expertise, and past experience (Mikaelian et al., 2014).

Table 4-2 Identification of and Rationale for Preclinical Endothelial Biomarkers for Vascular Injury

Predominant Specificity	Gene/ Target Symbol	Gene/Target Name and other symbols/names	Rationale
Endothelial Markers	Angpt2	Angiopoietin-2 ANG-2	The strategy to identify biomarkers of endothelial cell (EC) activation/damage was guided by the significant body of literature that describes EC activation/damage ultrastructurally and mechanistically in CDIV as well as in animal models. Biomarkers of EC damage are anticipated to lack specificity for DIVI, because EC activation also occurs in other diseases or toxicities, most notably inflammation and cancer. The activation of ECs is associated with leukocyte rolling and adhesion mediated primarily by the expression on ECs of vascular cell adhesion molecule 1 (VCAM1), intercellular adhesion molecule 1 (ICAM1), and E-selectin (SELE). The initial phase of the DIVI response is followed by a phase of angiogenesis involving pro- and antiangiogenic signals. In response to the production of endothelial-derived nitric oxide (NO), ECs produce EDN1, which causes vasoconstriction and proliferation of vascular smooth muscle cells (VSMCs). This proliferative response is balanced by the release of antiangiogenic factors, including prostacyclin that causes vasodilation, angiopoietin 2 (ANGPT2) that is released in the presence of vascular endothelial growth factor A (VEGFA) from the Weibel–Palade bodies of ECs, and thrombospondin 1 (TSP-1). TSP-1 is not specific to endothelial cells and also may be associated with markers of inflammation (Lopez-Dee et al. 2011).
	Edn1	Endothelin-1 ET-1	
	Sele (rat), Elam1	E-selectin (rat), endothelial leukocyte adhesion molecule 1 Soluble CD62 E	
	TSP-1	Thrombospondin-1 Thbs-1, Tsp1	
	Vegfa	Vascular endothelial growth factor, alpha	

Table 4-3 Identification of and Rationale for Preclinical Smooth Muscle Biomarkers for Vascular Injury

Predominant Specificity	Gene/ Target Symbol	Gene/Target Name and other symbols/names	Rationale
Smooth Muscle	Cnn1	Calponin-1 Calponin H1, smooth muscle	Most candidate VSMC biomarkers were selected based on their high expression in VSMCs. Their expression is reported to decrease in VSMC in the prototypical vascular injury (VI), a process that is anticipated to be concurrent with their leakage into the circulation. These candidate biomarkers are ACTA2, smoothelin, TGLN, CNN1, and h-CALD1. The expression of some of these proteins is influenced by the maturity and degree of hypertrophy of VSMCs. In response to the altered redox state of the tissues, caveolin 1 (CAV1) is produced and released from ECs, pericytes, and VSMCs leading to eNOS downregulation, which leads to vasoconstriction. Unfortunately, the development of assays for these candidate biomarkers in rats was unsuccessful thus far.

Table 4-4 Identification of and Rationale for Preclinical Inflammation Biomarkers for Vascular Injury

Predominant Specificity	Gene/ Target Symbol	Gene/Target Name and other symbols/names	Rationale
Inflammation	Timp1	Inhibitor of metalloproteinases 1 Tissue inhibitor of metalloproteinase 1	Candidate biomarkers for the inflammatory component of DIVI are released primarily by inflammatory cells in response to tissue stimulation/damage and remodeling. However, the source of some inflammation biomarkers is not clear, as they may be released by cells other than the inflammatory cells. For example, interleukin 6 (IL6) may be released by ECs. Many inflammation-related markers were considered with the understanding that inflammation biomarkers are not specific for DIVI.
	Lcn2	lipolcalin 2 Neutrophil gelatinase-associated lipocalin (NGAL); Alpha-2-microglobulin-related protein,	
	Cxcl1	Growth regulated alpha protein Cytokine-induced neutrophil chemoattractant 1, C-X-C motifchemokine 1 (KC/Gro/Cinc)	
	Agp1	Alpha-1 acid glycoprotein 1 Rat orosomucoid-1 (Orm1)	
	tNO	Total nitric oxide NO	

4.5.2 Clinical Biomarkers

Cytokines and other biomarkers of inflammation, such as C-reactive protein (CRP) and interleukin 6 (IL6), have been reported to be elevated in patients with vasculitis (Maksimowicz-McKinnon, et al., 2004) as well as during DIVI. However, these biomarkers are not specific to the vasculature, and therefore should not be used in the absence of biomarkers more specific to vascular injury. In addition to inflammatory factors, endothelial-related biomarkers and specific smooth muscle proteins constitute other promising biomarkers of vascular injury. By combining biomarkers of inflammation along with those reflective of damage to different vascular compartments, the consortium expects to qualify a panel of translational biomarkers allowing sensitive and specific detection and monitoring of DIVI. After an extensive literature review, the WP4 group initially selected approximately 80 biomarker candidates that were likely to be associated with the three main features involved in DIVI: damage to vascular endothelium, damage to smooth muscle, and inflammation. This list was then filtered on the basis of various criteria that were used to score the biomarker candidates, including the existence of published data in preclinical and clinical settings, parameters related to the feasibility of appropriate sampling or of large scale biomarker measurement, and intellectual property status. This approach resulted in the biomarkers that are listed in Table 4-5, Table 4-6Identification of and Rationale for Clinical Smooth Muscle Biomarkers of Vascular Injury 4-6 and Table 4-7. Supportive text and references in the Rationale column come from a recent review by Bendjama et al. (2014).

Table 4-5 Identification of and Rationale for Clinical Endothelial Biomarkers of Vascular Injury

Predominant Specificity	Gene/ Target Symbol	Gene/Target Name and other symbols/names	Rationale
Endothelial Markers	SELE	E-selectin	Changes in the expression of adhesion molecules such as increases in ICAM-1, VCAM, soluble E-selectin and P-selectin are early biomarkers of EC activation. Higher levels of adhesion molecules have been associated with various clinical entities as well as with DIVI. Adhesion molecules have been shown to be expressed in response to shear stress and to activation of tumor necrosis factor (TNF) pathways. Increases in ESM-1 and VEGF levels have been correlated with the level of neovascularization of tumors and may be useful in detecting the perivascular neovascularization. At this stage vessels also display chronic changes and significant fibrosis. Fibrosis is a physiologic healing process characterized by remodelling of the tissue through catalysis of existing matrix and collagen deposition. Candidate biomarkers selected to monitor these stages include proteins involved in this remodelling process, such as tissue inhibitor of metalloproteinase 1 (TIMP-1), thrombospondin 1 (TSP-1), endocan (ESM-1) and VEGF. Thrombomodulin has been reported to be a biomarker of vascular endothelial cell injury (Li et al, 2006)
	SELP	P-selectin	
	sICAM-1	Soluble Intercellular adhesion molecule 1	
	sVCAM-3	Soluble Vascular cellular adhesion molecule 3	
	THBD	Thrombomodulin	
	VEGF	Vascular endothelial growth factor	

Table 4-6 Identification of and Rationale for Clinical Smooth Muscle Biomarkers of Vascular Injury

Predominant Specificity	Gene/ Target Symbol	Gene/Target Name and other symbols/names	Rationale
Smooth Muscle	CALD1	H-Caldesmon	In some cases, the acute phase of DIVI can include the development of necrotic lesions in the vascular smooth muscle. In this stage one would expect to see leakage of tissue-specific molecules into the circulation, enabling the detection of the necrotic damage. Specific biomarkers of the smooth muscle—H-caldesmon (CALD1), H1-calponin (CNN1), smooth muscle alpha actin (ACTA2), smoothelin (SMTN) and transgelin (TAGLN)—were selected as specific biomarkers of the smooth muscle damage. These proteins are involved in the structural organization and regulation of the contractile complex and have the potential to serve as a sensitive and specific signal of smooth muscle necrosis both in DIVI and in human vascular disease. This concept is supported by elevated serum levels of smooth muscle troponin-like protein levels in patients with aortic aneurysm and aortic dissection.
	CNN1	Calponin-1 Calponin H1, smooth muscle	

Table 4-7 Identification of and Rationale for Clinical Inflammation Biomarkers of Vascular Injury

Predominant Specificity	Gene/ Target Symbol	Gene/Target Name and other symbols/names	Rationale
Inflammation	TIMP1	Inhibitor of metalloproteinases 1 Tissue inhibitor of metalloproteinase 1	There are numerous reports of increased levels of inflammatory factors in both DIVI and in clinical vasculitides. Inflammatory changes would be reflected by secretion of factors such as monokine induced by gamma interferon (MIG), interferon gamma induced protein 10 (IP10), stromal cell-derived factor 1 (SDF-1), IL6, CRP, macrophage inflammatory protein 1 alpha (MIP-1α), interleukin 8 (IL8), monocyte chemotactic protein 1 (MCP-1), and macrophage inflammatory protein 3 beta (MIP-3β) that promote the recruitment of inflammatory cells in the vascular wall and in the perivascular space.
	GROa	Cytokine- induced neutrophil chemoattractant-1 (KC, Cinc, CXCL1)	
	NGAL	lipolcalin 2 Neutrophil gelatinase-associated lipocalin	
	sICAM-3	Soluble Intercellular adhesion molecule 3	
	IL-6	Interleukin 6	
	IL-8	Interleukin 8	
	CRP	C-reactive protein	
	IP-10	Interferon gamma induced protein 10 / CXCL10	
	I-TAC	Interferon-Inducible T-Cell Alpha Chemoattractant / CXCL11	
	MCP-1	Monocyte chemotactic protein 1 / CCL2	
	MIG	Monokine induced by gamma interferon / CXCL9	
	SAA	Serum amyloid A	
	MIP-1A	Macrophage inflammatory protein 1 alpha / CCL3	

5 Preclinical Studies

5.1 Methods

5.1.1 Study design

Individual studies were conducted to support the qualification of proposed rat preclinical biomarkers of vascular injury (VI). The performance of the VI biomarker(s) was analyzed against the histological endpoints of acute VI. VI was defined by the histopathology of injury and vascular compartment affected, of which the focus was an acute degenerative process with or without inflammation that affected the intima, the media, and/or the adventitia of the vessels. This was achieved by using serum samples from rats treated with selected vascular toxicants or vasoactive, but not vasotoxic, compound(s) (negative control(s)).

Known vascular toxicants belonging to different pharmacological classes previously shown to induce vascular lesions in rats and which have been commonly used as tool compounds in investigative studies aimed at identifying mechanisms and biomarkers for DIVI were used for the qualification studies (Table 5-1). In addition, because many of the selected compounds are vasoreactive, a vasoactive (but non-vasotoxic compound), yohimbine, was assessed as a negative control to test candidate biomarker specificity.

5.1.1.1 Rationale for Species/Strain, Route of Administration and Dose Selection

The rat was selected, because it is a standard species recognized by both US and international regulatory agencies for use in safety evaluation studies. The preferred rat strain is the Crl:CD(SD) because of the knowledge of this strain's general pathology, and because DIVI has been previously observed and is well characterized in this strain. However, other strains may be used as needed because of strain-specific sensitivity to compounds causing DIVI, or based upon the familiarity of the PSTC contributors.

The route of administration for each selected compound varied and depended on the route previously known to induce DIVI in rats following administration of the selected compound.

5.1.1.2 Test System and Management

Crl:CD(SD) rats were approximately 8-12 weeks of age and weighed approximately 180–400 grams at dose initiation. Rats were housed according to standard guidelines of each sponsor, in a controlled environment (64-79 °F, 30-70% relative humidity), with a 12-hour light/dark cycle. Rats were acclimated to local housing conditions for at least 7 days following study group assignment and were offered a certified rodent diet and filtered tap water *ad libitum*, as determined by each respective sponsor. Food was withheld overnight before collection of blood samples for clinical pathology and before scheduled necropsy.

5.1.1.3 Experimental Groups

Clinically acceptable animals were allocated to dose groups, following the review of pretreatment data (body weights, clinical observations) and using a computer assisted randomization procedure based on body weights.

Four to six rats/group/time-point were used. Control rats received an equivalent volume of respective vehicle through the same route of administration as did the treatment groups.

5.1.1.4 Dosing Regimen

A dosing regimen for each selected vascular toxicant or negative control was chosen based on findings of earlier studies in which Crl:CD(SD) rats were administered doses that resulted in DIVI (vascular toxicant) or for negative control samples with yohimbine, a dose that is known to be vasoactive but has not previously been associated with VI (Table 5-1).

The route of administration, treatment duration, and necropsy/sample collection time points were compound-dependent based on historical data and follow the general guidelines as indicated below.

General post-dose necropsy/sample collection time point guidelines:

tA- Approximately one half the time expected to induce VI (observed histologically)

tB- Approximate known onset of VI (observed histologically in ~ ≥ 50% of the animals with moderate severity was the aim based on relevant literature/experience)

tC- Approximately 24-48 hours following the known onset of VI to ensure VI onset is not missed.

tRec- Approximately 30 days following the last dose administered (recovery)

5.1.2 Compound selection rationale (i.e., compound(s) triggering the organ injury under evaluation)

Vascular toxicants belonging to different pharmacological classes previously shown to induce vascular injury in rats, and which have been commonly used as tool compounds in investigative studies aimed at identifying mechanisms and biomarkers for DIVI, were used for preclinical studies to support biomarker qualification (see Table 5-1). In addition, a vasoactive but non-vasotoxic compound, yohimbine, was assessed as a negative control to evaluate candidate biomarker specificity.

Table 5-1 Preclinical studies to enable qualification of DIVI biomarkers

Compound	Mechanism	Animals per Group	Route / Vehicle	Number of Doses Administered	Timepoints Analyzed (Hours post dose)				Expected predominant vessel compartment(s) affected	Expected vessel size/type affected	Expected inflammation type/ localization
					tA	tB	tC	Recovery (tRec)			
IL2	IL-2 receptor	5	Subcutaneous/ sterile water	1/day	48	120	ND	168	Endothelium	All	Mixed, perivascular
Fenoldopam	DA1 receptor	5	Subcutaneous/ Saline	1	6	24	72	168	Smooth muscle, endothelium[1]	Medium arteries, arterioles	Mixed, perivascular
SK&F95654	PDE3 inhibitor	6	Subcutaneous/ DMSO	1	16	24	48	720	Smooth muscle, endothelium[1]	Medium arteries, Arteriole	Mixed, perivascular
CI-1044	PDE4 inhibitor	6	Oral Gavage/ 0.5% Methylcellulose	1	16	24	48	720	Smooth muscle, endothelium[1]	Medium arteries, arteriole	Mixed, perivascular
Yohimbine (negative control)	Alpha 2-adrenoreceptor antagonist	6	Oral Gavage/ 0.5% Methylcellulose	1	8	16	24	720	None	None	None
Compound X	unknown	5	Oral Gavage/ 0.5% Methylcellulose	1	8	16	24	720	Smooth muscle	Medium arteries	None
Midodrine	α1-receptor agonist	6	Oral Gavage/ 0.5% Methylcellulose	1	4	16	48	720	Smooth muscle, endothelium[1]	Medium arteries, Arteriole	Mixed, perivascular
Nicorandil	K+ATP channel opener	5	Oral Gavage/ Saline	1/day	24	48	96	NA	Smooth muscle, endothelium[1]	Medium arteries, Arteriole	Mixed, perivascular

[1] Some literature suggests that endothelial cell stimulation (i.e., response to shear stress) is the pivotal early event leading to smooth muscle injury (Mikaelian et al. 2014).

5.1.3 Histopathology

All studies were performed at a single institution (Pfizer), except for the IL2 study (Roche), with tissues collected and processed according to their respective Standard Operating Procedures (SOPs). Briefly, collected tissues were fixed in 10% Neutral Buffered Formalin (NBF), except for the testes, which were fixed in Modified Davidson's fixative prior to transfer to NBF. The mesentery, kidney, heart, liver, lung skeletal muscle and testis were collected across all studies. All tissues were processed onto a slide (embedded in paraffin wax, sectioned, and stained with hematoxylin and eosin). Light microscopic examination of all tissues collected was performed on all animals, except for those found dead on study, using the PSTC VIWG Lexicon and guidance (Mikealian et al. 2014). Microscopic examination was performed blinded to the biomarker and clinical pathology data, but not to the study group in which a targeted blind approach was used as needed (Burkhardt et al. 2011). A pathology peer review was conducted for the nicorandil, IL2, and fenoldopam studies, and a consensus opinion was reached between the two pathologists.

5.1.4 Biomarker assays

Assay validation protocols for the preclinical biomarker assays followed the 2012 Best-Practices set forth by the PSTC (Critical Path Institute: https://pstcworkspace.c-path.org/BestPractices/PSTC-AssayValidationGuidance-FINAL-20120705.doc). Quality control samples (blank, positive, negative controls) were evaluated and assay performance goals were set to ensure the generation of reliable data for each biomarker. All biomarkers are ELISA-based, met criteria for fit-for-purpose assay validation, and, as appropriate, were transferred with validation to two CROs for future use.

The following parameters were included in the validation protocols:

1. working range of the assay
2. intra- and inter-assay precision
3. accuracy/recovery
4. limits of quantitation
5. dilutional linearity
6. sample freeze/thaw and storage stability
7. analytical interference

5.1.5 Statistical analysis

For the exploratory studies, the statistical analysis is to complement the univariate analysis performed for the clinical studies. A multivariate approach and more detailed analysis of injury by compartment and morphology (ie. injury type) will be performed after completion of the confirmatory study set.

Briefly, the endpoint for injury used here is "any vascular injury." The "injection site injury," which included vascular injury, from the IL-2 study was of similar incidence and severity for animals with subcutaneous administration of vehicle or IL-2 and was therefore not considered an endpoint against which to compare biomarker analysis for DIVI.

We set biomarker values exceeding the limits of quantification to the upper limit of quantification for that assay or to half of the lower limit of quantification for that assay.

We derived Receiver Operating Characteristic (ROC) curves and the area under those curves (AUC) as the scoring metric. We used % as a unit for the AUC. Biomarkers for which the distribution for animals with VI was the same as the distribution for animals without VI had a value of 50%. If all biomarker values for animals with VI were higher than the values for animals without VI, the AUC was 100%. If all biomarker values for animals with VI were lower than the values for animals without VI, the AUC was 0%.

We computed AUC for each of three given time points as well as a recovery time-point for each compound. We also computed an over time score across the time-points tA, tB and tC (excluding recovery). For the overall score to be computed, there had to be > 5 animal with VI among time points. For the by time-point analysis, there needed to be more than one animal with VI for the AUC to be computed.

5.2 Results

5.2.1 Assay validation

Table 5-2, Table 5-3, and Table 5-4 summarize the preclinical assay validation studies that were completed. Detailed assay validation documents are available upon request.

5.2.2 Summary of results

Table 5-5 shows a Summary by study and time point of histopathology and biomarker results. The primary histopathology changes are described with those in parenthesis indicating a minor contribution to the DIVI morphology. A toxicologically meaningful biomarker response is considered > 80% AUC increase or < 20% AUC decrease from control animal values. The detailed statistical analysis results are in Appendix 9.1.

Table 5-2 Assay Validation data for final candidate rat endothelial biomarkers

Predominant Specificity	Gene / Target Symbol	CRO	Validation Status	Matrix	Endog Corr. ? [a]	Quantitation Range[b]	Accuracy[c] %RE range	Precision[c] %CV range	MRD[e]	QC1[f]	QC2	QC3	Vol?	Mean Endogenous in Validation (+/-30%)
Endothelial Markers	Angpt2	MPI	Complete	K3-EDTA plasma	Yes	1.61 – 3500 ng/mL	-0.7 to -9	4 to 10	1:35	22.3	2.47	0.28	20 uL	4.9 – 9.1 ng/mL
				Serum	Yes	1.61 – 3500 ng/mL	-9 to -0.2	4 to 12	1:35	22.3	2.47	0.28	20 uL	6.7 – 12.3 ng/mL
	Edn1 (ET-1)	Covance	Complete	K3-EDTA plasma	No	3.12 - 200 pg/mL	4 to 17	8 to 11	1:2	33.3	8.33	2.08	150 uL	4.5 – 8.4 pg/mL
				Serum	Yes	3.12 - 200 pg/mL	10 to 19	5 to 8	1:2	33.3	8.33	2.08	150 uL	7.8 – 14.6 pg/mL
	Sele (rat), Elam1	MPI	Complete	Plasma										
				Serum		0.098 – 200 ng/mL	-8 to 4	3 to 6	1 :4	76.1	23.3	5.3	30 uL	
	TSP-1	Covance	Complete	K2-EDTA plasma	Yes	2970 – 76700 ng/mL	-6 to -14	12	1 :100	753	83.7	na	30 uL	2284-4242 ng/mL
				Serum	Yes	2970 – 37700 ng/mL	-20 to -14	10-11	1 :100	na	83.7	na	30 uL	15200-28230 ng/mL
	Vegfa	MPI	Complete	K2-EDTA plasma	Yes	93.9 – 96000 pg/mL	-17 to -8	3 to 5	1:3	20k	1250	78.1	30 uL	105 – 195 pg/mL
				Serum	Yes	93.9 – 96000 pg/mL	-18 to -8	4 to 5	1:3	20k	1250	78.1	30 uL	126 – 234 pg/mL

[a]Results corrected for detectable endogenous analyte; [b]The quantitation range corrected for Minimum Required Dilution; [c]Accuracy: % RE of repeated analysis of analyte spiked into matrix; [d]Precision: % CV of repeated analysis of analyte spiked into matrix; [e]Minimum Required Dilution; [f]QC values not corrected for MRD. Multiply by MRD for absolute value.* BQ

Table 5-3 Assay Validation data for final candidate rat smooth muscle biomarkers

Predominant Specificity	Gene / Target Symbol	CRO	Validation Status	Matrix	Endog Corr. ? [a]	Quantitation Rangeb	Accuracyc %RE range	Precisionc %CV range	MRDe	QC1f	QC2	QC3	Vol?	Mean Endogenous in Validation (+/-30%)
Smooth Muscle	Cnn1	Covance	Failed	Plasma										
				Serum										

[a]Results corrected for detectable endogenous analyte; [b]The quantitation range corrected for Minimum Required Dilution; [c]Accuracy: % RE of repeated analysis of analyte spiked into matrix; [d]Precision: % CV of repeated analysis of analyte spiked into matrix; [e]Minimum Required Dilution; [f]QC values not corrected for MRD. Multiply by MRD for absolute value.* BQL

Table 5-4 Assay Validation data for final candidate rat inflammation biomarkers

Predominant Specificity	**Gene / Target Symbol**	**CRO**	**Validation Status**	**Matrix**	**Endog Corr. ?** [a]	**Quantitation Range** [b]	**Accuracy** [c] %RE range	**Precision** [c] %CV range	**MRD** [e]	**QC1** [f]	**QC2**	**QC3**	**Vol?**	**Mean Endogenous** in Validation (+/-30%)
Inflammation	Timp1	Covance	Complete	Plasma	Yes	5.67 – 687 ng/mL	-8 to 6	3 to 5	1:100	2.19	0.24	0.08	30 uL	5*-9 ng/mL
				Serum	Yes	5.67 – 687 ng/mL	-10 to 3	5-8	1:100	2.19	0.24	0.08	30 uL	5*-9 ng/mL
	Lcn2	Covance	Complete	K2-EDTA plasma	Yes	3.3 – 2760 ng/mL	-4 to -5	4 to 9	1:100	11.8	1.31	0.44	30 uL	150-270 ng/mL
				Serum	Yes	3.3 – 2760 ng/mL	-6 to 2	5 to 8	1:100	11.8	1.31	0.44	30 uL	150-280 ng/mL
	Cxcl1 (KC/Gro)	MPI	Complete	K2-EDTA plasma	Yes	16.46 - 36000 pg/mL	-5 to 12	3 to 5	1:2	12000	444.44	49.38	30 uL	36 – 66 pg/mL
				Serum	Yes	16.46 - 36000 pg/mL	-14 to -4	6 to 8	1:2	12000	444.44	49.38	30 uL	150 – 278 pg/mL
	Agp1	Covance	Complete	Plasma	Yes	7.8 – 500 mcg/mL	-4	4 to 8	1:5000	0.020	na	na	30 uL	23 - 43 mcg/mL
				Serum	Yes	7.8 – 500 mcg/mL	-3	5 to 6	1:5000	0.020	na	na	30 uL	22 – 41 mcg/mL
	tNO	Covance	Complete	K3-plasma	Yes	12.5 - 400 umol/L	-15 to -4	5 to 16	1:2	150	37.6	9.4	150 uL	21 umol/L
				Serum	Yes	18.8 - 600 umol/L	-8 to 4	5 to 7	1:3	150	37.6	9.4	100 uL	21 umol/L

[a]Results corrected for detectable endogenous analyte; [b]The quantitation range corrected for Minimum Required Dilution; [c]Accuracy: % RE of repeated analysis of analyte spiked into matrix; [d]Precision: % CV of repeated analysis of analyte spiked into matrix; [e]Minimum Required Dilution; [f]QC values not corrected for MRD. Multiply by MRD for absolute value.* BQL

Table 5-5 Summary of Preclinical Microscopic Findings and Statistical Results

Compound	Time point	Primary VI histopathology using lexicon categories (additional changes)	Incidence of VI by time point (VI+/total rats per group)	Endothelial Biomarkers with meaningful response (↑/↓) by time point	Inflammation Biomarkers with meaningful response (↑/↓) by time point
Nicorandil	tA	no VI	0/5	no statistical analysis	no statistical analysis
	tB	inflamm mono/mixed	3/5	↓ VEGFa	↑ LCN2, TIMP1
	tC	and# EC H/H	5/5	↓ Angpt2	↑ NO, MCP1, TIMP1, AGP1
	tRec	n/a	n/a	n/a	n/a
Fenoldopam	tA	EC H/H	1/5	no statistical analysis	no statistical analysis
	tB	and# inflamm mono (SM D/N)	5/5	↑ Angpt2, VEGFa; ↓ SELE	↑ LCN2, MCP1, TIMP1, AGP1
	tC	and# SM D/N	5/5	↓ SELE	↑ LCN2, TIMP1, AGP1
	tRec	EC H/H; SM H/H; SM D/N; inflamm mono (mixed)	5/5	↓ SELE	↑ TIMP1, AGP1
Cl-1044	tA	inflamm mixed	2/6	↑ Angpt2, SELE, ET1	↑ MCP1, TIMP1, AGP1
	tB	inflamm mixed	3/6	↑ Angpt2, ET1; ↓ SELE	↑ LCN2, TIMP1, AGP1
	tC	(and# SM D/N)	6/6	↑ Angpt2, ET1	↑ LCN2, TIMP1, AGP1
	tRec	inflamm mono	¼	no finding	no finding
SK&F 95654	tA	no VI	2/6	↑ SELE, Angpt2	↑ LCN2
	tB	SM D/N	6/6	↑ Angpt2; ↓ ET1, TSP-1,	↑ LCN2, TIMP1
	tC	and# inflamm mixed (SM hyal/hyperos)	6/6	↓ Angpt2; ↓ ET1, TSP-1	↑ MCP1, TIMP1, AGP1
	tRec	EC H/H; SM hyal/hyperos; SM H/H	1/6	↓ ET1, TSP-1; ↑ VEGFa,	↓ LCN2, TIMP1

Midodrine	tA	SM D/N	5/5	↑ Angpt2, TSP-1	↑ NO, Cxcl1, LCN2, MCP1, TIMP1
	tB	SM D/N	5/5	↑ Angpt2, SELE, ET1	↑ NO, Cxcl1, LCN2, MCP1, TIMP1
	tC	and[#] inflamm mixed	4/4	↑ SELE, ET1, VEGFa	↑ NO, LCN2, MCP1, TIMP1
	tRec	SM H/H; SM D/N	1/6	no finding	↑ NO, Cxcl1, MCP1, TIMP1
Compound X	tA	no VI	0/4	no finding	no finding
	tB	no VI	0/4	no finding	no finding
	tC	SM D/N	2/4	↓ Angpt2; ↑ TSP1, VEGFa	↑ NO, LCN2
	tRec	no VIs	0/5	no finding	no finding
IL2	tA	EC H/H; inflamm mixed; SM Hyal/Hypereos	6/6	↑ Angpt2, SELE, VEGFa	↑ LCN2, MCP1, TIMP1
	tB	EC H/H; inflamm mixed; SM Hyal/Hypereos	6/6	↑ Angpt2, SELE, VEGFa	↑ NO, LCN2, MCP1, TIMP1
	tC	n/a	n/a	n/a	n/a
	tRec	EC H/H; inflamm mixed; SM Hyal/Hypereos	6/6	↑ Angpt2,	↓ AGP1
Yohimbine (negative control)	tA	no VI	0/5	no finding	no finding
	tB	no VI	0/5	no finding	no finding
	tC	no VI	0/5	no finding	no finding
	tRec	no VI	0/5	no finding	no finding

[#]"and" indicates that the histopathology at that time point include the finding(s) at the previous time point(s). *IV injection site excluded, as similar severity and morphology occurred in concurrent vehicle control animals. VI: vascular injury; tRec: time point in recovery phase; Inflamm: inflammation; EC: vascular endothelial cell; SM: vascular smooth muscle; mono: mononuclear leukocyte infiltrate; mixed: mixed leukocyte infiltrate; D/N: degeneration, necrosis, apoptosis; H/H: hypertrophy, hyperplasia; Hyalin/Hypereos: hyalinization, hypereosinophilia; n/a not applicable because no sample.

5.3 Future studies

5.3.1 Implications of results for preclinical – clinical translation strategy

Translational support for the clinical markers comes from studies in non-clinical animal models using candidate biomarkers that are expected to reflect changes in the same three primary histologic features of vascular injury in animals and humans. The non-clinical biomarkers include biomarkers of inflammation (tNO, Agp1, Cxcl1 (KC/Gro), Lcn2, TIMP1) and endothelial cell injury (Angpt2, Edn1 (ET-1), VEGFa, TSP-1, E-selectin); their performance was analyzed by correlation to histopathologic endpoints of acute DIVI in rats. We note that while there are some differences between the non-clinical and clinical markers, we expect that their pathophysiologic response to drug-associated injury will be similar given the similar histologic features of vascular injury in animals and humans. Also note that for both the preclinical and clinical work to date, assays for the smooth muscle proteins (h1-calponin, H-caldesmon, SM alpha actin, SM22/transgelin, smoothelin) were not technically feasible to design or did not meet validation requirements, but are included in the document for future consideration as technologic advancement may allow.

5.3.2 Proposed preclinical studies to address gaps or to expand qualification

Planned non-clinical studies in rat will include testing of candidate markers in “confirmatory” studies using additional compounds, as well as selected repeat compounds from the initial “exploratory” studies, and a balloon angioplasty study to complement the mechanical injury model evaluated in the clinical qualification. The approach of two “tiers” that uses comparable study designs and biomarker assays will enable refinement of the biostatistical plan, increased robustness of biostatistical assessment across two independent study sets with bridging compounds, and a diversity to evaluate the biomarkers in response to the histopathologic pattern rather than mechanism of injury. In addition, biomarker analysis of studies to assess the specificity of the markers in preclinical studies using compounds that do not impact the vasculature but that cause injury in other organs is also planned. Finally, development of assays and generation of opportunistic data from retrospective (analysis of archived samples) or prospective studies in support of developing DIVI biomarkers in dogs and monkeys are also planned.

6 Clinical Studies

6.1 Methods

6.1.1 Study design (i.e., each study description)

SAFE-T selected a series of surrogate populations presenting histopathologic lesions that are morphologically similar to, or comparable in type or vascular compartment with, preclinical DIVI. Human conditions used in this strategy span a wide variety of vessel sizes and types; however, by focusing the analysis on the three main histopathologic features (endothelial damage, smooth muscle damage, and inflammation), the translational strategy can be applied

to all of them regardless of vessel type or mechanism of injury. This strategy is based on the hypothesis that damage to similar compartments of blood vessels is likely to result in an overlapping biomarker signature. To cover most of the histopathologic features found in preclinical DIVI, and in the interest of rapid enrollment for both the exploratory and confirmatory studies, SAFE-T selected patients presenting with different diseases involving vessels of different types and sizes, affecting predominantly different areas of the vascular compartments, and characterized by different localization and cell type of inflammatory infiltrates. As such, patients with large vessel vasculitides (Takayasu's arteritis), medium vessel lesions (patients undergoing balloon/stent angioplasty), or small-sized vessel vasculitides (i.e., hypersensitivity vasculitis, and mixed cryoglobulinemia affecting arterioles, capillaries and venules) were included; with different type of vessels affected such as arteries (Takayasu's arteritis, patients undergoing balloon/stent angioplasty) and veins (Behçet's disease); with injury predominantly to the endothelium (hypersensitivity vasculitis, mixed cryoglobulinemia, Behçet's disease), media (patients undergoing balloon/stent angioplasty), or adventitia (Takayasu's arteritis). In addition, the selection of patients undergoing balloon angioplasty was based on the goal of including a population with acute injury to the vessel wall and endothelium. Although one of the original goals of the SAFE-T project for DIVI biomarkers was to include "negative" drug challenge studies, and feedback from EMA and FDA recommended inclusion of "positive" drug challenge studies, there was not enough time or budget to complete these studies.

6.1.2 Biomarker assays

The SAFE-T Consortium has established common standard operating procedures for the validation of assays, with the measurement of samples and bioanalytical activity performed in a quality-controlled environment. The consortium performed the validation of assays based on a "fit-for-purpose" approach as previously described (Lee et al. 2006; Lee et al. 2007; Lee and Hall 2009) by proceeding in discrete stages of validation of increasing stringency. The assays were characterized in two steps: first, an initial technical validation during the exploratory phase, followed by an extensive validation during the confirmatory phase. Assay performance was assessed by estimating assay dynamic range (limit of detection, lower and upper limit of quantification), precision (intra-assay precision, intermediate precision), linearity, recovery, short-term stability and freeze-and-thaw cycle stability in the matrices of interest (serum, lithium-heparin and EDTA plasma).

The SAFE-T Consortium focused on the MesoScale Discovery (MSD) multiplexed immunoassay format, in addition to the standard sandwich ELISA. The assays on the different formats were available from third party vendors or developed on the basis of monoclonal antibodies developed by partners within the SAFE-T consortium.

The primary issue that we have been working to address relates to the analytical platform used to measure the DIVI biomarkers. The "exploratory" data shared with the agencies in April 2015 was generated from 467 sampled analyzed using three MSD multiplexed assays (called Panel 1 [a 4-plex], Panel 2 [a 4-plex], and a custom 9-plex) whose format was called the Ultrasensitive Assay (UA). However, before the independent "confirmatory" samples could be analyzed using the same kits, MSD changed the format of the assay for Panel 2 (also known as the Human Vascular Injury Panel, a commercially available kit) from UA to a newer "V-PLEX" format in an attempt to improve the performance and utility of the kit. In

addition, reagents for six of the nine biomarkers on the 9-plex kits also changed from UA to V-PLEX.

As a result of the change in kit formats for Panel 2 and the 9-plex, and the inconsistent performance of the different lots of the UA 9-plex, SAFE-T was left with two non-comparable data sets. One set of data was from 467 exploratory samples generated in 2013 on the UA versions of Panel 1 (4-plex), Panel 2 (4-plex), and the 9-plex, and a second set of data was from 301 samples (called Confirmatory) generated in 2015 on a new lot of the UA Panel 1, V-PLEX Panel 2, and V-PLEX 9-plex.

Numerous statistical and experimental approaches have been attempted by SAFE-T to bridge the two data sets (exploratory samples & confirmatory samples); however, neither correction factors nor predictive statistical models have as yet given SAFE-T the confidence that we can appropriately integrate/bridge the two data sets for the purpose of DIVI biomarker qualification.

6.1.3 Clinical data management

Clinical Data Management was performed by Koehler eClinical for all studies with an electronic case report form (eCRF) defined in OpenClinica.

Data Management included the cleaning process for the variables to be analyzed. In addition, data from several external sources were mapped to the clinical eCRF data. Thus, biomarker results were directly transferred from the screening laboratories to Data Management for mapping. For analysis, relevant data were mapped to Clinical Data Interchange Standards Consortium (CDISC) / Study Data Tabulation Model (SDTM) format for analysis.

6.1.4 Statistical analysis

During the exploratory phase, various descriptive statistics and exploratory statistical methods were used to determine the performance of individual biomarkers and combinations thereof to differentiate patients with vascular injury from non-vascular injury subjects. A tabular summary of the objectives and the statistical methods used is shown in Table 6-1. All statistical calculations were performed using the R statistical language (R Foundation, 2012). For biomarker data with concentrations that are below the lower limit of quantification (LLOQ), the LLOQ was divided by two. All biomarker data were log2-transformed before statistical analysis. For the purpose of building class-prediction models, the Classification for MicroArrays (CMA) package (Slawski et al, 2008) was used. Based on previous analyses showing that the Random Forest algorithm produced better results, this method was applied using 10-fold cross validation. Univariate discriminative performances based on the area under ROC curve (AUROC) was assessed using the R package ROCR for all defined comparisons.

To determine the optimum number of markers each model should contain, the biomarkers were ranked according to their variable importance score (VIS) as calculated by the Random Forest algorithm. A graph of the AUROC vs the number of biomarkers (added according to their rank of the VIS) in the discrimination model was then generated. These curves typically show a steep initial increase and then levels off showing a broad plateau with very similar AUROC values regardless of the number of markers. The minimal number of biomarkers needed to reach the level of the plateau was identified.

Table 6-1 presents the various descriptive statistics and exploratory statistical methods used to determine the performance of individual biomarkers and combinations.

Table 6-1 Statistical methodologies used in the various studies

Dataset summary and analysis	Tabulated data	Figure / Table
Univariate descriptive statistics of biomarkers	Min, Max, Mean, Q1, Median, Q3, 95th percentile, SD, IQR	Tables with the values of the descriptive statistics, Individual AUROC per biomarker
Identification of discrimination models and marker combinations for: -Active vasculitis vs HV -Angioplasty time baseline vs 24h post-BA	Random Forest Algorithm using 10-fold cross-validation. Variable selection using the variable importance score of the Random Forest method	AUROC vs number of biomarkers for the discriminations indicated, A ROC Curve with the number of biomarker chosen and the confusion matrix

6.2 Results

6.2.1 Assay validation

Between the Exploratory and the Confirmatory phase, the provider of the MSD kits changed the antibody clones for several biomarkers. A comparison (correlation) between the old and the new version (Vplex) of MSD kit was made to verify the reproducibility of the generated data during the exploratory phase. Thus, old samples (n = 37), for which data were generated with the old version, were measured again using the new version (Vplex) of the kit. The Pearson correlation coefficients are shown in Table 6-2.

Table 6-2 Correlations between biomarker levels measured with two different versions of assay/kit

Biomarker	Panel 1 Old vs New	Panel 2 Old vs New	9-plex Old vs New
CRP		0.96	
ICAM-1		0.343	
SAA		0.94	
VCAM-1		0.447	
E-selectin	0.755		
P-selectin	0.745		
ICAM-3	0.398		
THBD	0.371		
Gro-a			0.482
IL-6			0.704
IL-8			0.876
IP10			0.477
ITAC			0.672
MCP-1			0.479
MIG			0.529
MIP1-A			0.753
VEGF			0.739

Among the 17 biomarkers tested, 9 showed a Pearson r correlation coefficient below 0.7. Only two biomarkers (CRP and SAA) showed a Pearson r correlation coefficient above 0.9. According to these correlation data, with multiple biomarkers showing poor correlations, it was not possible to bridge the Exploratory and the Confirmatory phase data sets. Therefore, we used only on the confirmatory phase data to inform the biomarker qualification.

6.2.2 Statistical analysis

6.2.2.1 Input data

For the comparison between Healthy Volunteers and patients with vasculitis, we used data of 32 vasculitis patients in active phase and 32 age- and gender-matched healthy volunteers. For the second analysis, we compared the data of 55 patients undergoing a balloon angioplasty before and approximately 24-hrs after the procedure.

6.2.2.2 Descriptive statistics

Healthy Volunteers (HV)

A summary of biomarker levels in healthy volunteers can be found in Table 6-3. All values are in pg/ml (without log2 transformation).

Vasculitis Active (Vasc)

A summary of the biomarker measurements in patients with active vasculitis can be found in Table 6-4. All values are in pg/ml (without log2 transformation).

Balloon Angioplasty Baseline (BA1)

A summary of biomarker levels at baseline in patients undergoing balloon angioplasty procedure can be found in Table 6-5. All values are in pg/ml (without log2 transformation).

24h post-Balloon Angioplasty (BA2)

A summary of biomarker measurements in patients 24-hr after undergoing balloon angioplasty can be found in Table 6-6. All values are in pg/ml (without log2 transformation).

Table 6-3 Summary of biomarker levels in healthy volunteers

Biomarker	Minimum	5th Percentile	25th Percentile	Median	75th Percentile	95th Percentile	Maximum	Mean	Standard Deviation	Interquartile Range
CRP	67	173	563	1268	2953	8018	42047	2604	4802	2390
GROA	7.2	13.1	29.6	48.0	67.7	112.5	170.1	52.3	30.8	38.2
ICAM1	145	226	280	361	442	573	980	374	123	161
INTLK6	0.18	0.18	0.18	0.52	0.66	1.18	2.27	0.54	0.36	0.48
INTLK8	2.6	4.1	6.7	8.3	11.7	18.6	47.5	9.7	5.4	5.0
IP10	38.7	89.6	128.0	163.9	222.1	528.3	1685.4	215.8	181.5	94.1
ITAC	9.8	9.8	30.5	41.3	60.8	97.5	292.5	50.9	41.3	30.3
MCP1	8.9	162.0	244.7	318.8	378.2	481.0	720.6	316.9	107.7	133.4
MIG	22.0	35.1	73.0	110.6	173.5	337.2	734.8	140.6	112.1	100.6
MIP1A	1.0	5.1	7.9	10.5	13.6	26.7	118.7	12.6	11.1	5.7
SAA	131	665	1532	2908	6225	13998	58259	5026	6836	4694
SELE	2.4	3.6	5.6	8.7	11.1	16.8	20.5	9.1	4.1	5.5
SELP	3.6	25.6	39.2	50.4	63.2	81.1	107.8	51.3	18.3	24.0
SICAM3	0.10	0.25	0.33	0.41	0.51	0.69	1.03	0.43	0.14	0.18
THBD	1.65	2.19	2.81	3.37	3.89	4.84	5.91	3.38	0.81	1.08
VCAM1	274	402	522	622	759	991	1510	657	200	237
VEGF	11.2	41.2	77.0	130.6	222.4	404.8	1205.8	172.5	157.1	145.5

Table 6-4 Summary of biomarker levels in patients with active vasculitis

Biomarker	Minimum	5th Percentile	25th Percentile	Median	75th Percentile	95th Percentile	Maximum	Mean	Standard Deviation	Interquartile Range
CRP	296	335	852	3298	6506	54726	141820	13939	32799	5654
GROA	37.5	37.7	49.9	68.0	151.8	544.6	1144.0	165.2	257.9	101.8
ICAM1	224	240	321	578	626	786	1082	523	220	305
INTLK6	0.18	0.18	0.38	0.72	1.21	4.10	7.04	1.28	1.68	0.83
INTLK8	6.3	6.7	11.1	13.7	32.0	54.2	54.7	21.2	15.1	21.0
IP10	45.7	86.0	180.7	290.7	611.6	1860.8	2979.4	592.3	755.6	430.9
ITAC	9.8	9.8	29.2	56.4	139.9	426.9	528.6	121.9	148.4	110.7
MCP1	91.0	105.0	196.7	269.4	365.4	603.4	618.0	297.7	151.4	168.6
MIG	13.2	15.7	52.3	70.4	100.6	705.7	1559.7	197.5	366.5	48.3
MIP1A	4.5	5.6	6.6	13.5	27.7	48.4	53.8	20.0	16.0	21.2
SAA	414	875	2004	3802	11544	246319	327622	36275	88519	9540
SELE	2.9	4.3	8.1	11.1	14.5	27.1	30.1	12.5	7.3	6.3
SELP	31.4	39.3	46.6	57.9	62.4	82.7	136.6	58.6	22.0	15.8
SICAM3	0.22	0.24	0.30	0.39	0.43	0.70	1.08	0.43	0.20	0.12
THBD	2.3	2.4	2.6	3.1	3.8	5.4	6.3	3.5	1.1	1.2
VCAM1	339	443	550	800	1081	2287	2309	969	586	531
VEGF	48.2	68.5	124.4	181.1	254.6	389.7	724.9	217.3	147.6	130.2

Table 6-5 Summary of biomarker levels at baseline in patients before undergoing balloon angioplasty (BA1)

Biomarker	Minimum	5th Percentile	25th Percentile	Median	75th Percentile	95th Percentile	Maximum	Mean	Standard Deviation	Interquartile Range
CRP	255	437	1305	2815	6367	23072	38236	5917	8168	5062
GROA	7.3	22.0	53.5	74.0	96.0	201.0	404.3	90.1	72.0	42.5
ICAM1	209	235	361	495	606	851	1227	509	210	245
INTLK6	0.18	0.18	0.52	0.94	1.30	2.90	4.89	1.16	0.98	0.78
INTLK8	4.7	12.7	26.2	42.6	73.7	178.8	272.1	62.5	58.4	47.4
IP10	53.0	68.0	114.0	150.7	222.2	430.0	472.8	187.9	112.3	108.2
ITAC	9.8	9.8	9.8	24.1	40.1	79.5	166.7	30.4	28.6	30.3
MCP1	73.0	104.1	241.5	307.0	361.5	484.8	1058.4	315.6	149.5	120.0
MIG	31.5	39.8	65.0	123.9	212.4	384.4	806.6	159.2	133.7	147.5
MIP1A	8.0	10.1	31.2	49.3	121.2	436.8	622.5	109.9	139.6	90.0
SAA	339	1208	2764	4673	10263	28932	116075	10127	17735	7499
SELE	3.6	4.7	7.1	8.4	10.8	15.5	21.5	9.2	3.3	3.7
SELP	22.2	32.1	45.2	55.4	68.1	90.7	108.3	58.0	18.0	22.9
SICAM3	0.23	0.27	0.41	0.50	0.60	0.83	3.61	0.58	0.49	0.19
THBD	1.76	1.98	2.60	2.95	3.96	8.15	11.36	3.55	1.81	1.36
VCAM1	404	501	652	823	989	1519	3782	919	496	338
VEGF	1.0	34.1	215.2	391.4	903.5	1733.6	2432.6	628.3	573.7	688.4

Table 6-6 Summary of biomarker levels in patients 24-hr after balloon angioplasty (BA2)

Biomarker	Minimum	5th Percentile	25th Percentile	Median	75th Percentile	95th Percentile	Maximum	Mean	Standard Deviation	Interquartile Range
CRP	67	324	1193	2457	4724	21824	39303	5259	7930	3532
GROA	2.4	6.2	11.0	15.3	27.5	83.0	167.2	26.6	30.7	16.5
ICAM1	12	80	316	436	558	828	1257	450	224	242
INTLK6	0.18	0.18	0.56	0.78	1.24	4.70	5.55	1.23	1.27	0.68
INTLK8	2.2	3.4	6.0	10.7	25.3	116.3	216.1	25.6	40.3	19.3
IP10	85.7	118.7	223.1	339.4	574.8	2749.7	5631.4	655.9	1024.1	351.8
ITAC	9.8	9.8	9.8	9.8	14.8	33.5	69.4	15.2	12.2	5.1
MCP1	47.9	60.8	105.8	138.4	181.0	333.8	493.3	156.8	83.7	75.2
MIG	53.2	93.4	210.7	524.2	763.9	2154.0	4319.0	703.6	890.2	553.2
MIP1A	5.8	8.3	14.8	27.2	61.3	340.3	655.6	76.7	122.2	46.4
SAA	35	314	2537	3768	7332	24813	166445	11766	30687	4795
SELE	2.2	3.9	6.1	7.6	10.5	12.7	16.6	8.1	3.0	4.4
SELP	5.2	13.6	19.0	26.6	35.0	52.2	70.0	29.3	13.1	16.1
SICAM3	0.07	0.23	0.34	0.46	0.57	0.83	3.27	0.53	0.45	0.23
THBD	0.37	1.76	2.45	2.82	3.71	5.84	9.81	3.26	1.62	1.27
VCAM1	45	140	623	767	898	1579	2750	816	426	275
VEGF	0.97	0.97	0.97	3.54	6.16	160.70	381.52	22.88	72.93	5.19

6.2.2.3 Univariate ROC Curve Analysis

In Table 6-7 the univariate ROC curve analysis of individual biomarkers for the discrimination of healthy volunteer and active vasculitis patients is shown. In Table 6-8 the univariate ROC curve analysis of individual biomarkers for baseline and 24-hr post-balloon angioplasty is shown.

Table 6-7 Individual AUROC data for the discrimination healthy volunteer vs active vasculitis

Biomarker	AUROC
CRP	0.749
GROA	0.774
ICAM1	0.774
INTLK6	0.751
INTLK8	0.796
IP10	0.691
ITAC	0.589
MCP1	0.466
MIG	0.436
MIP1A	0.681
SAA	0.621
SELE	0.707
SELP	0.623
SICAM3	0.533
THBD	0.644
VCAM1	0.731
VEGF	0.736

Table 6-8 Individual AUROC data for the discrimination baseline (BA1) versus 24-hr post Balloon Angioplasty (BA2)

Biomarker	AUROC
CRP	0.456
GROA	0.112
ICAM1	0.422
INTLK6	0.494
INTLK8	0.190
IP10	0.799
ITAC	0.303
MCP1	0.146
MIG	0.850
MIP1A	0.361
SAA	0.444
SELE	0.416
SELP	0.089
SICAM3	0.434
THBD	0.455
VCAM1	0.428
VEGF	0.061

6.2.2.4 Predictive Modeling

The plateau of the AUROC was reached at about 5 biomarkers (Figure 6-1). Therefore, the top 5 ranked biomarkers (Table 6-9) were used to construct the model.

Figure 6-1 **Predictive performance as a function of the number of biomarkers for the discrimination of healthy volunteers (HV) versus patients with active vasculitis (Vasc)**

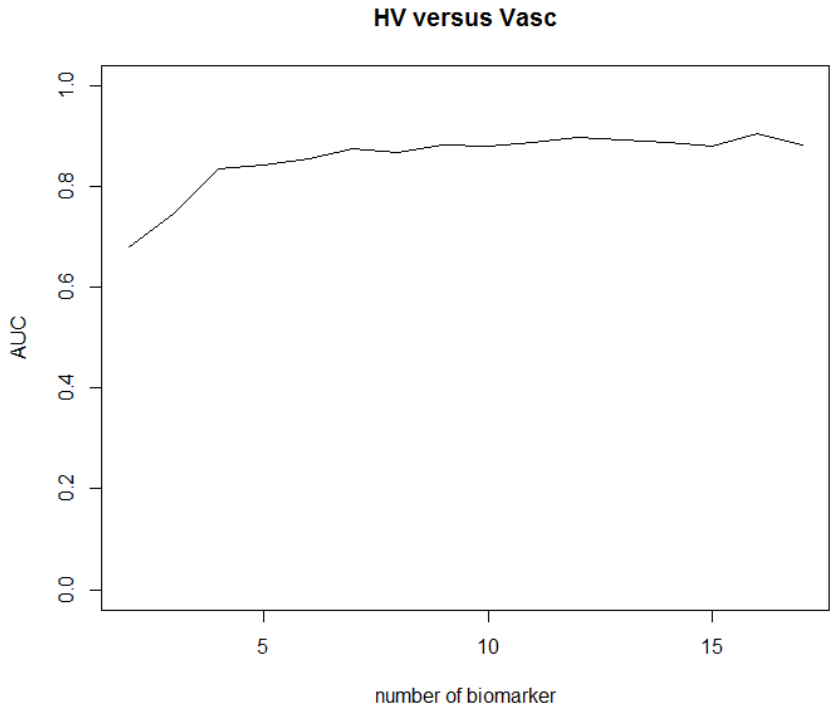
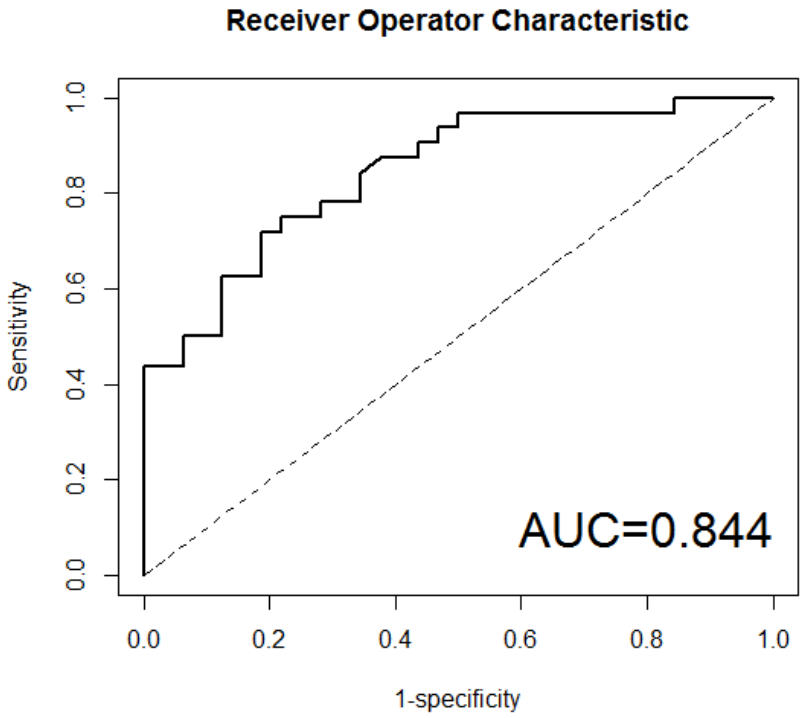


Table 6-9 **Ranking of the biomarkers according to their marginal added value in the predictive model in Figure 2.**

Biomarker	Rank
INTLK8	1
GROA	2
INTLK6	3
CRP	4
ICAM1	5

We used a Random Forest model to discriminate between the two populations (Figure 6-2). The Random Forest method was also used to select the variables. Note that the list of biomarkers includes one endothelial biomarker, providing some specificity of the predictive model to the vasculature.

Figure 6-2 **ROC curve for the discrimination of healthy volunteers (HV) versus patients with active vasculitis. A Random Forest model with five biomarkers was used.**



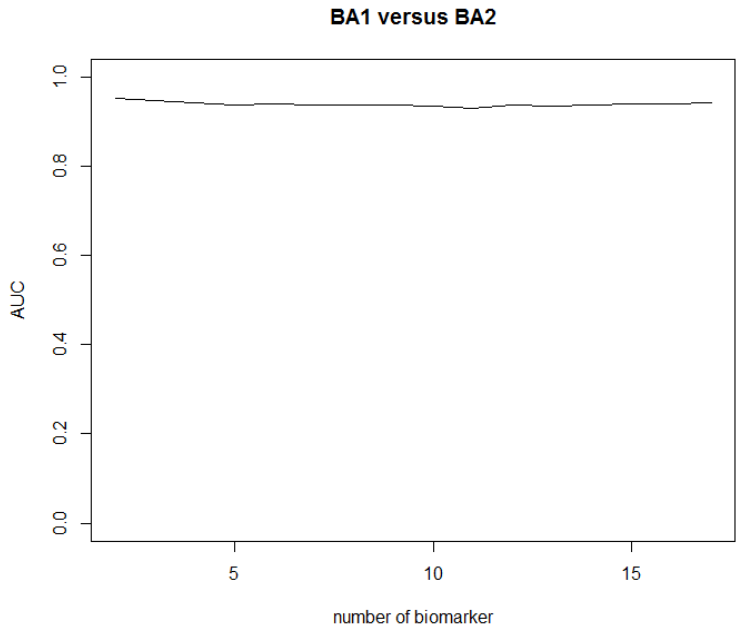
The positive outcome is defined as the patients of the HV group (Table 6-10 and Figure 6-3).

Table 6-10 **Predictive performance statistics of a random forest model for the discrimination of healthy volunteers (HV) from patients with active vasculitis (Vasc)**

	HV predicted	Vasc predicted
HV true	24	8
Vasc true	8	24

Statistic measured	Result
Accuracy	0.750
Sensitivity	0.750
Specificity	0.750

Figure 6-3 **Predictive performance as a function of the number of biomarkers for the discrimination of baseline (BA1) versus 24-hr post Balloon Angioplasty (BA2)**



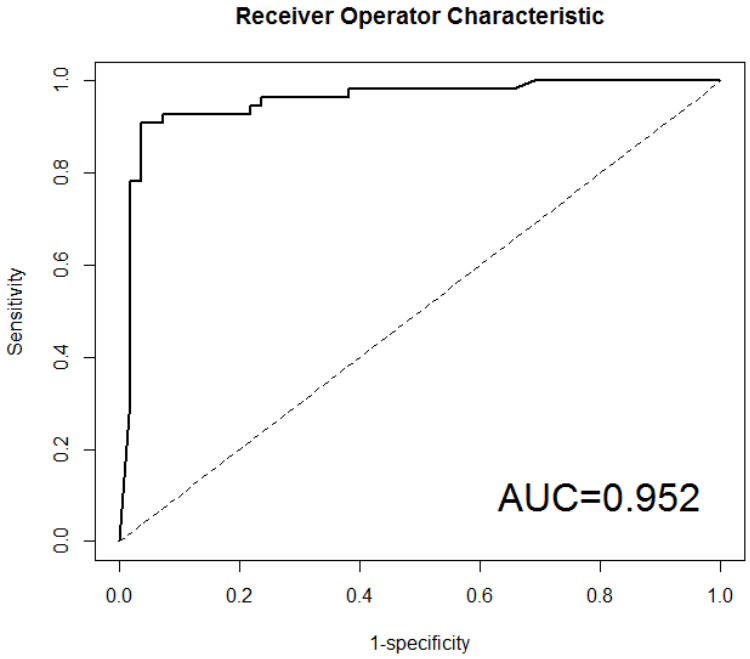
The curve is very flat, indicating that very few biomarkers are sufficient for a good discrimination. We chose 2 biomarkers to construct the model (Table 6-11).

Table 6-11 **Ranking of the biomarkers according to their marginal added value in the predictive model in Figure 4.**

Biomarker	Rank
VEGF	1
SELP	2

We use a Random Forest model to discriminate between the two populations. The Random Forest method is also used to select the variables (Figure 6-4). In this case, the predictive model is based solely on endothelial biomarkers and does not include biomarkers of inflammation.

Figure 6-4 **ROC curve for the discrimination of baseline (BA1) versus 24-hr post Balloon Angioplasty (BA2). A Random Forest model with 2 biomarkers was used.**



The positive outcome is group BA1 (Table 6-12).

Table 6-12 Predictive Statistics of Random Forest Model for Discrimination of Baseline (BA1) from 24-hr post Balloon Angioplasty (BA2)

	BA1 predicted	BA2 predicted
BA1 true	48	7
BA2 true	4	51

Statistic measured	Result
Accuracy	0.900
Sensitivity	0.927
Specificity	0.873

7 Conclusion

7.1 Summary of key findings supporting the qualification request

The supportive information for the candidate biomarkers listed in the FDA LoS and the EMA LoS comes from both the literature and from experimentally derived data. The literature evidence was summarized in Section 4.5, wherein the rationale for identifying each of the biomarkers as "candidates" is outlined, along with references to support their inclusion in the qualification process. Experimental data supporting the non-clinical and clinical biomarkers are included in Section 6.2 and Section 6.2.

A consistent interpretation of the clinical results is that combinations of vascular injury biomarkers can discriminate healthy volunteers from patients with disease, and with significantly better performance than individual biomarkers. For the biomarkers that are being analyzed using a multivariate Random Forest model, there are indeed several statistical approaches that can be used to rank the biomarkers according to their "importance" or contribution to the predictive performance of the model. However, there are several factors that make it premature to further narrow the biomarker list based on the current data set. First, as recently noted by the BQRT, the analytical validation performed to date has revealed "broad precision profiles" of the biomarker assays, which limit our confidence in the ability to generate comparable results in repeated measurements with the same platform. Also, as discussed by the BQRT, we are reporting "exploratory observations obtained only from a small sample subset" of our overall clinical study; more subjects are needed to confirm these responses once the analytical validation issue has been addressed. And finally, the biomarker profiles from our two clinical models of vascular injury (with vasculitides patients with an acute flare on chronic injury, and balloon angioplasty patients with a known onset of acute injury) are different; it is not yet clear how best to combine these profiles into a unified predictive model of vascular injury.

This summary data package is designed to provide the rationale from the literature and the experimental data collected to encourage the conduct of nonclinical and exploratory clinical analyses to evaluate the translational relevance of changes in the expression of candidate DIVI biomarkers reported in this document. Moreover, data sharing and integrating data across trials can foster an accelerated path for numerous drug development programs. In light of the

circumstances outlined above, the data we have generated remain promising, and FDA on November 7, 2016, and EMA on November 7, 2017, issued a LoS to provide a useful mechanism to encourage others in the field to address gaps and facilitate the ultimate goal of clinical biomarker qualification.

8 Literature References

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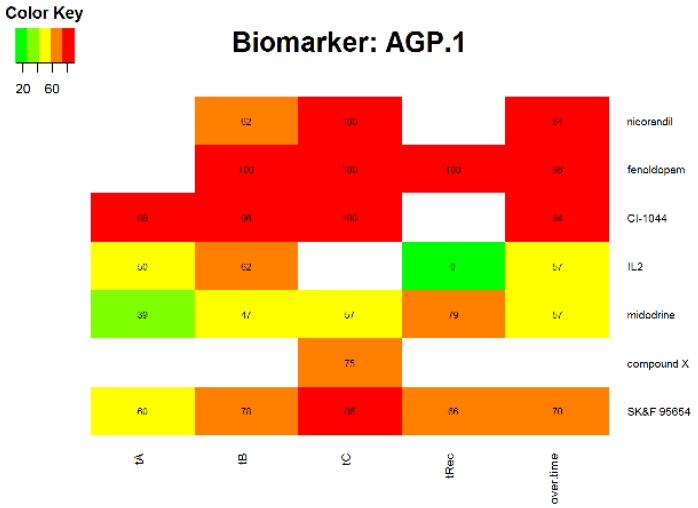
9 Appendices

9.1 Preclinical biomarker AUC results

Biomarker AUC heat map graphs and table generated from preclinical exploratory studies. Biomarkers for which the distribution for animals with VI was the same as the distribution for animals without VI had a value of 50%. If all biomarker values for animals with VI were higher than the values for animals without VI, the AUC was 100%. If all biomarker values for animals with VI were lower than the values for animals without VI, the AUC was 0%. A toxicologically meaningful biomarker response is considered a > 80% AUC (red box) or a < 20% AUC (dark green box) with respect to control animal values. In the AUC heat maps and table, “over.time” refers to an analysis that combined data from time points tA, tB and tC.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IL2
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compound X
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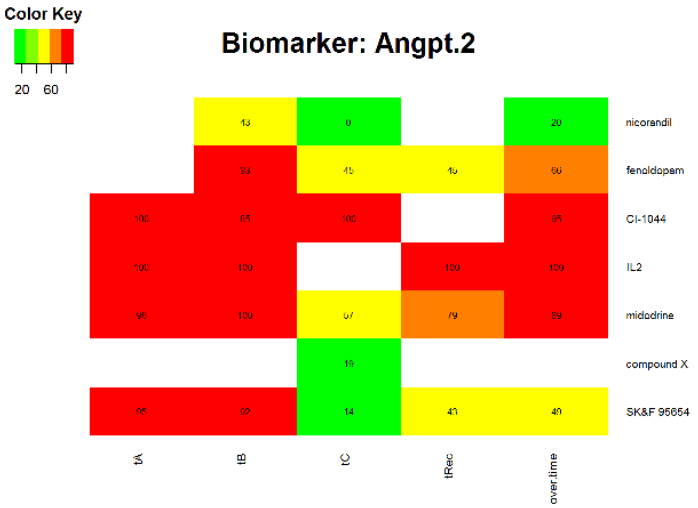
tA
tB
tC
tRec
over.time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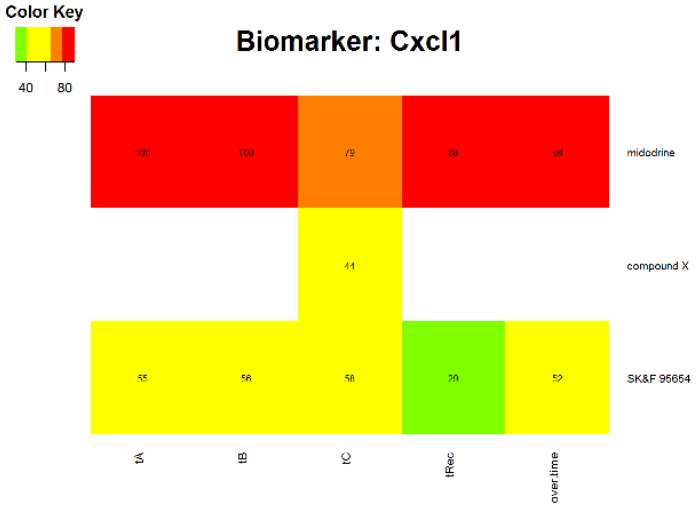
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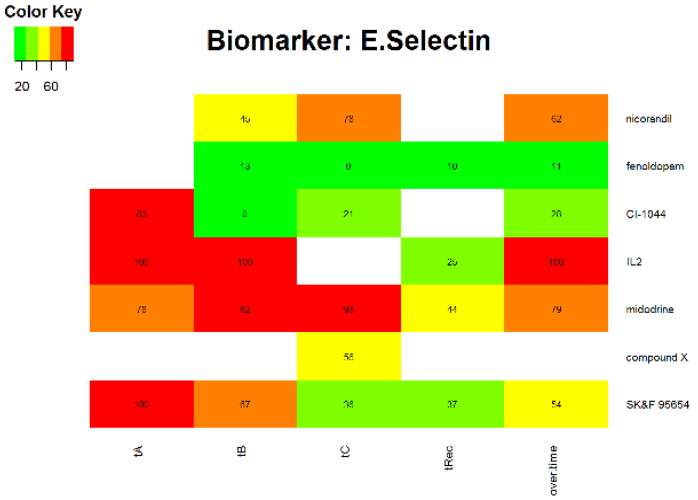
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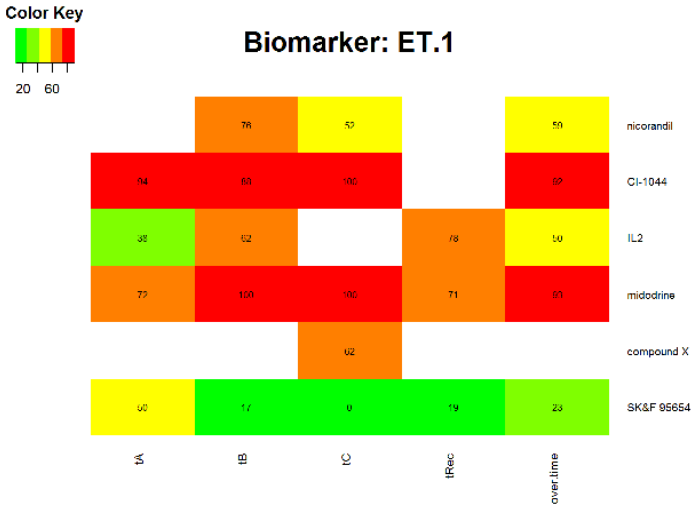
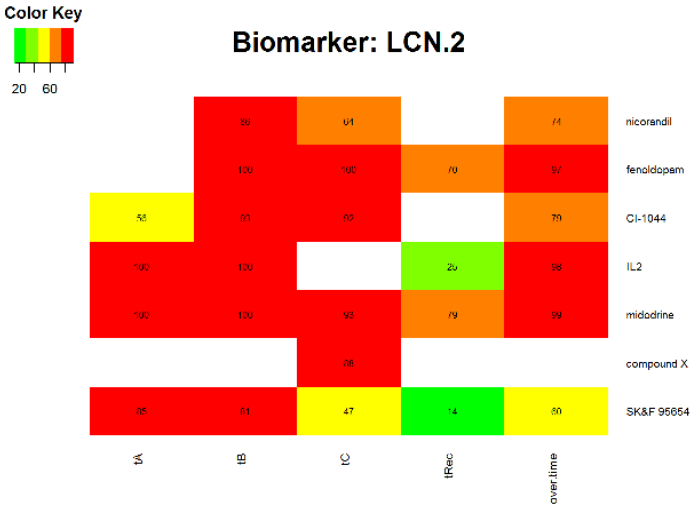
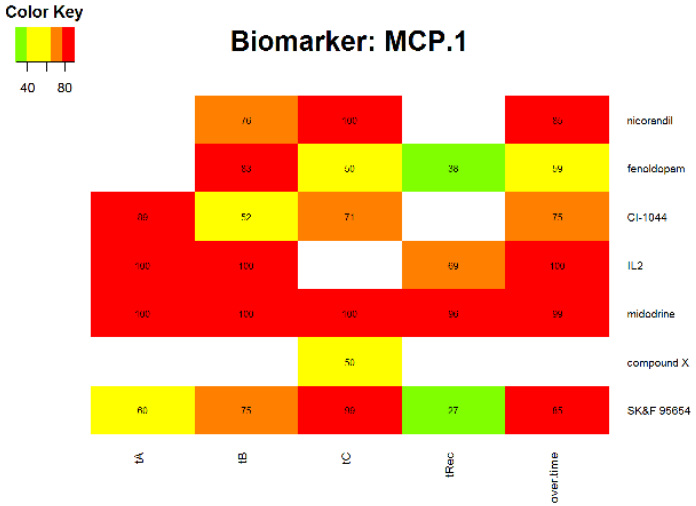
midodrine
compound X
SK&F 95654
tA
tB
tC
tRec
over.time


Color Key
20 60
Biomarker: ET.1
nicorandil
CI-1044
IL2
midodrine
compound X
SK&F 95654
tA
tB
tC
tRec
over.time


Color Key
20 60
Biomarker: LCN.2
nicorandil
fenoldopam
CI-1044
IL2
midodrine
compound X
SK&F 95654
tA
tB
tC
tRec
over.time


Color Key
40 80
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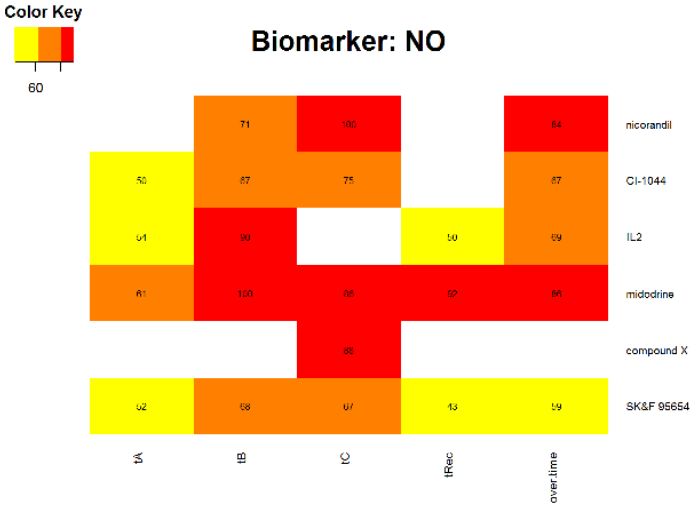
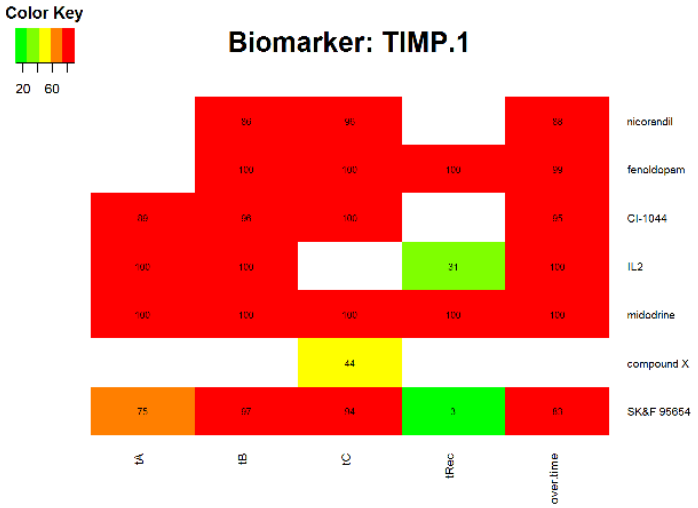
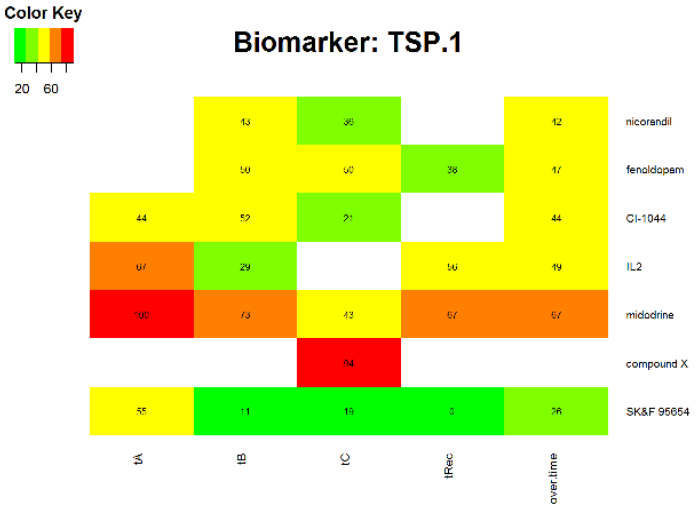
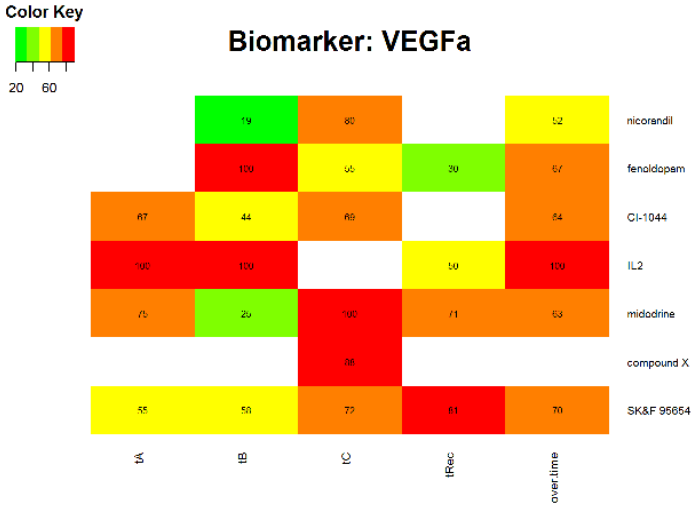
nicorandil
fenoldopam
CI-1044
IL2
midodrine
compound X
SK&F 95654
tA
tB
tC
tRec
over.time


Color Key
60
Biomarker: NO
nicorandil
CI-1044
IL2
midodrine
compound X
SK&F 95654
tA
tB
tC
tRec
over.time


Color Key
20 60
Biomarker: TIMP.1
nicorandil
fenaldopam
CI-1044
IL2
midodrine
compound X
SK&F 95654
tA
tB
tC
tRec
over.time


Color Key
20 60
Biomarker: TSP.1
nicorandil
fenaldopam
CI-1044
IL2
midodrine
compound X
SK&F 95654
tA
tB
tC
tRec
over.time


Color Key
20 60
Biomarker: VEGFa
nicorandil
fenaldopam
CI-1044
IL2
midodrine
compound X
SK&F 95654
tA
tB
tC
tRec
over.time

AUC Tables

The table below has the AUCs that are displayed in the heat maps for reference.

AUC Values

Biomarker	Compound	Time points				
		tA	tB	tC	tRec	over.time
AGP.1	nicorandil		62	100		84
AGP.1	fenoldopam		100	100	100	98
AGP.1	CI-1044	89	96	100		94
AGP.1	IL2	50	62		0	57
AGP.1	midodrine	47	42	57	79	58
AGP.1	compound X			75		
AGP.1	SK&F 95654	60	78	86	66	70
Angpt.2	nicorandil		43	0		20
Angpt.2	fenoldopam		93	45	45	66
Angpt.2	CI-1044	100	85	100		95
Angpt.2	IL2	100	100		100	100
Angpt.2	midodrine	100	100	57	79	92
Angpt.2	compound X			19		
Angpt.2	SK&F 95654	95	92	14	43	49
Cxcl1	nicorandil					
Cxcl1	fenoldopam					
Cxcl1	CI-1044					
Cxcl1	IL2					
Cxcl1	midodrine	100	58	79	88	90
Cxcl1	compound X			44		
Cxcl1	SK&F 95654	55	56	58	29	52
E.Selectin	nicorandil		45	78		62
E.Selectin	fenoldopam		13	0	10	11
E.Selectin	CI-1044	83	0	21		28
E.Selectin	IL2	100	100		25	100
E.Selectin	midodrine	82	92	93	44	83
E.Selectin	compound X			56		
E.Selectin	SK&F 95654	100	67	36	37	54
ET.1	nicorandil		76	52		59
ET.1	fenoldopam					

ET.1	CI-1044	94	88	100		92
ET.1	IL2	38	62		78	50
ET.1	midodrine	100	83	100	71	96
ET.1	compound X			62		
ET.1	SK&F 95654	50	17	0	19	23
LCN.2	nicorandil		86	64		74
LCN.2	fenoldopam		100	100	70	97
LCN.2	CI-1044	56	93	92		79
LCN.2	IL2	100	100		25	98
LCN.2	midodrine	100	100	93	79	99
LCN.2	compound X			88		
LCN.2	SK&F 95654	85	81	47	14	60
MCP.1	nicorandil		76	100		85
MCP.1	fenoldopam		83	50	38	59
MCP.1	CI-1044	89	52	71		75
MCP.1	IL2	100	100		69	100
MCP.1	midodrine	100	100	100	96	99
MCP.1	compound X			50		
MCP.1	SK&F 95654	60	75	99	27	85
NO	nicorandil		71	100		84
NO	fenoldopam					
NO	CI-1044	50	67	75		67
NO	IL2	54	90		50	69
NO	midodrine	100	100	86	92	98
NO	compound X			88		
NO	SK&F 95654	52	68	67	43	59
TIMP.1	nicorandil		86	96		88
TIMP.1	fenoldopam		100	100	100	99
TIMP.1	CI-1044	89	96	100		95
TIMP.1	IL2	100	100		31	100
TIMP.1	midodrine	100	100	100	100	100
TIMP.1	compound X			44		
TIMP.1	SK&F 95654	75	97	94	3	83
TSP.1	nicorandil		43	36		42
TSP.1	fenoldopam		50	50	38	47
TSP.1	CI-1044	44	52	21		44

TSP.1	IL2	67	29		56	49
TSP.1	midodrine	73	25	43	67	53
TSP.1	compound X			94		
TSP.1	SK&F 95654	55	11	19	0	26
VEGFa	nicorandil		19	80		52
VEGFa	fenoldopam		100	55	30	67
VEGFa	CI-1044	67	44	69		64
VEGFa	IL2	100	100		50	100
VEGFa	midodrine	25	50	100	71	54
VEGFa	compound X			88		
VEGFa	SK&F 95654	55	58	72	81	70