

Longitudinal Meta-cohort study protocol using systems biology to identify vaccine safety biomarkers

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Abbreviations: AEFI, Adverse Event Following Immunization; AESI, Adverse Event of Special Interest; VITT, Vaccine-Induced Immune Thrombocytopenia and Thrombosis; TTS, Thrombosis with Thrombocytopenia Syndrome; INSIS, The International Network of Special Immunization Services; PF4, Anti-platelet Factor 4; ITP, Immune Thrombocytopenic Purpura; GBS, Guillain-Barré Syndrome; SAE, Serious Adverse Event; HIT, Heparin Induced Thrombocytopenia; MACE, Major Adverse Cardiac Events; LVEF, Left Ventricular Dysfunction; RSV, Respiratory Syncytial Virus; PBMC, Peripheral Blood Mononuclear Cells; MERS-CoV, Middle East Respiratory Syndrome Coronavirus; HCoV, Human Coronavirus; PRR, Pattern Recognition Receptor; SIC, Special Immunization Clinic Network; AEFI-CAN, Australian Adverse Event Following Immunization-Clinical Assessment Network; CISA, US Clinical Immunization Safety Assessment Network; GVDN, Global Vaccine Data Network; ALIVE, African Leadership in Vaccinology Expertise Network; PVP, Precision Vaccines Program; MVRG, Mayo Clinic Vaccine Research Group; OPBG, Bambino Gesù Children's Hospital; UBC, University of British Columbia; SickKids, The Hospital for Sick Children; SAB, Scientific Advisory Board; ICMJE, International Committee of Medical Journal Editors; CDC, Centers for Disease Control and Prevention; WHO, World Health Organization; LMIC, Low and Middle Income Country; NIH, United States National Institutes of Health; DMAC, Data Management and Analysis Core; NPI, Nominated Principal Investigator; NMO, Network Management Office; SPEAC, Safety Platform for Emergency Vaccines; LC-MS, Liquid Chromatography coupled to tandem Mass Spectrometry; HPLC, High-Performance Liquid Chromatography; DIA, Data Independent Acquisition; PEA, Proximity Extension Assay; BCAA, Branched-Chain Amino Acid; RP, Reverse Phase; UPLC-MS, Ultra-Performance Liquid Chromatography-Mass Spectrometry; ESI, Electrospray Ionization; HILIC, Hydrophilic Interaction Liquid Chromatography; MRM, Multiple Reaction Monitoring; IVTT, In Vitro Transcription and Translation; CyTOF, Mass Cytometry Time of Flight; GWAS, Genome-Wide Association Studies; GSA, Global Screening Array; MHC, Major Histocompatibility Complex; GATK, Genome Analysis Toolkit; MOFA, MultiOmics Factors Analysis; DIABLO, Data Integration Analysis for Biomarker Discovery using Latent Components; BTM, Blood Transcriptional Module; FDR, False Discovery Rate; GEO, Gene Expression Omnibus; SOP, Standard Operation Procedure; CRF, Case Report Form; SPF, Sample Processing Form; MTAs, Material Transfer Agreements; DTUAs, Data Transfer Use Agreements; QC, Quality Control; QA, Quality Assurance.

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ABSTRACT

The International Network of Special Immunization Services (INSIS) was established to investigate the causes and risk factors of rare adverse events following immunizations (AEFIs) and develop immunization strategies for mitigating or preventing risk for individuals with prior AEFIs or at risk of AEFIs. INSIS integrates clinical data with multi-omic technologies (e.g., transcriptomics, proteomics, metabolomics) through a global consortium of clinical networks, leading immunology, pharmacogenomics teams to uncover the molecular mechanisms behind AEFIs. The network ensures accurate and standardized data collection and analysis through rigorous data management and quality assurance processes. INSIS also implements harmonized case definitions and protocols for collecting data and samples related to rare AEFIs, such as myocarditis, pericarditis, and Vaccine-Induced Immune Thrombocytopenia and Thrombosis (VITT) after COVID-19 vaccinations. This protocol outlines the comprehensive approach to enhance risk-benefit assessments of vaccines across populations, identify actionable biomarkers to inform discovery and development of safe vaccines, and support personalized vaccination strategies.

1. Introduction

Vaccines are among the most effective public health interventions, playing a critical role in preventing and controlling multiple public health emergencies and pandemics, such as influenza A/H1N1 in 2009, the Ebola outbreak in West Africa in 2014, COVID-19, and most recently monkey pox (mpox). Advances in biotechnology have allowed for the licensure of many new vaccines. However, similar to other medical interventions, they have potential risks. While serious adverse events following immunizations (AEFI)'s were recognized after introduction of smallpox and diphtheria antitoxin vaccinations in 18th and 19th century, respectively, the first scientific review on Hazards of Immunization, summarizing mostly case reports and case series, was only published in 1967 [1].

While much progress has been made since the 1990's, especially in high income countries, in early detection and quantification of vaccine risks using both passive and active surveillance for hypothesis generation and testing of AEFI's [2], there has been limited progress in understanding their biological and molecular mechanisms to mitigate or prevent newly detected vaccine risk [3]. The rarity of these serious vaccine risks, the relative immaturity of the underlying systems biology science, plus limited funding contributed to this limited progress.

COVID-19 vaccines were developed at unprecedented speed, receiving authorization under accelerated timelines and procedures within 10 months of the pandemic being declared [4,5]. These vaccines have been credited with preventing up to 20 million deaths from COVID-19 in the first year after their roll-out [6]. While the overwhelming benefits of COVID-19 vaccination continue to outweigh risks [7], rare AEFIs were identified through post-market surveillance [8]; two of which, Thrombosis with Thrombocytopenia Syndrome (TTS), including Vaccine-Induced Immune Thrombocytopenia and Thrombosis

(VITT) and myocarditis/pericarditis were most prominent. Additionally, increased risks of immune thrombocytopenic purpura (ITP) and Guillain-Barré Syndrome (GBS) have also been observed with adenoviral vector vaccines [9,10]. Given the ongoing use of adenoviral vector platforms in developing vaccines against emerging diseases, understanding the pathophysiology of VITT and other AEFIs associated with these vaccines remains a priority.

VITT has been linked to adenoviral vector COVID-19 vaccines (e.g., ChAdOx1 and Ad.26.COV-2.S). First reported in March 2021, VITT is characterized by rapidly progressive thrombosis, particularly in cerebral venous sinuses and splanchnic circulation veins, accompanied by thrombocytopenia and markedly elevated D-dimer and anti-platelet factor 4 (PF4) antibodies driving platelet activation [11–15]. The incidence of VITT ranges from 0.1 to 1.2 cases per 100,000 vaccinations following Ad26.COV-2.S and 1 to 5 per 100,000 following the first dose of ChAdOx1 vaccine [16]. Early diagnosis and treatment have reduced case fatality rates from as high as 40 % to 13–15 % [17].

Myocarditis and pericarditis emerged as safety signals following the rollout of COVID-19 mRNA vaccines. Although these vaccines have been pivotal in controlling the pandemic, there is an increased incidence of myocarditis and pericarditis, particularly among young males aged 12–29 years after the second dose [18,19–25]. Initial data suggested most patients appeared to recover quickly. Ongoing studies are focused on determining long-term outcomes, including resolution of cardiac MRI changes and cardiac-specific biomarkers, with some studies reporting persistence of MRI changes and symptoms in a subset of patients [26]. Importantly, despite these rare events, the benefits of COVID-19 vaccination in preventing severe disease, hospitalization, and death continue to far outweigh the risks. The mRNA platform is now being used to develop vaccines against a range of other diseases including influenza, Respiratory Syncytial Virus (RSV), Nipah, and Lassa fever, as well as for non-infectious indications such as cancer [11,27].

Myocarditis and pericarditis have also been reported following non-mRNA COVID-19 vaccines, including the adjuvanted protein subunit vaccine Novavax NVX-CoV2373 and AstraZeneca's ChAdOx1 [23].

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Early studies suggest that the spike protein may contribute to these rare cardiac events [28,29]. Ongoing research into the underlying biological mechanisms is essential to refine our understanding of vaccine safety. Continued surveillance and investigation are critical to ensuring public confidence and the safety of vaccination programs.

The International Network of Special Immunization Services (INSIS) was formed in 2020 to develop a “first-of-its kind” global network for investigating rare AEFIs, starting with AEFIs associated with COVID-19 vaccination [30]. When TTS/VITT and myocarditis emerged as vaccine safety signals in 2021, specialist clinical networks were engaged to develop guidance for clinical investigation and management [31]. In some cases, these networks were able to quickly collect clinical data and biosamples from patients in their care in real-time. Clinical immunization assessment networks, such as the Canadian Special Immunization Clinic (SIC) Network and Australian Adverse Event Following Immunization-Clinical Assessment Network (AEFI-CAN), mobilized to support these efforts and evaluate patients requiring additional COVID-19 vaccine doses to complete the series. The Global Vaccine Data Network (GVDN) also initiated a study of genetic markers of COVID-19 vaccine adverse events of special interest (AESI), with samples collected by several INSIS sites, including those associated with SIC Network and AEFI-CAN [32].

INSIS has collaborated with these networks to harmonize data and sample collection to conduct downstream systems biology analyses and support genomics studies via GVDN. This collaboration ensures a sufficient sample size for robust analyses, supports multi-omics analysis of the collected samples, and enhances inter-operability of data to enable larger and more impactful analyses, while expanding the potential for discovery and replication.

INSIS combines clinical investigation with immunologic studies and systems biology “adversomics” such as transcriptomics, proteomics, epigenetics, and metabolomics. Adversomics describes the use of omics and other technologies in studying adverse reactions related to vaccines. These technologies measure relevant categories of molecules (e.g., DNA, RNAs, proteins, metabolites) in a given sample investigating possible patterns related to vaccine recipients with adverse events versus those without such adverse events [33,34]. This comprehensive approach has the significant advantage of not making any assumptions regarding potentially relevant molecular mechanisms but rather casting a broad net to define molecular pathways associated with, and potentially contributing to, AEFIs.

Studying AEFIs is crucial because they negatively impact the health of those experiencing them as well as vaccine uptake and public confidence in vaccination programs [35]. Understanding the clinical spectrum, risk factors, and underlying mechanisms of AEFIs informs the risk-benefit assessment of vaccines [36]. Additionally, these insights are essential for the discovery and development of safe vaccines, particularly as new platforms are employed to protect against emerging diseases. Together, these approaches will enable more informed decision-making, ultimately contributing to better public health outcomes. The infrastructure and methodologies developed by INSIS can be leveraged for future studies of other AEFIs for different vaccines against current and emerging pathogens or novel vaccines for non-infectious indications (e.g., vaccines against cancer, allergy or drug overdose).

In this report, we describe INSIS’ current approach and methodology, including the recruitment of participants from multiple global sites, harmonization of data collection, and use of advanced laboratory techniques to analyze biological samples. This also highlights the importance of understanding AEFIs to inform vaccine safety, improve public confidence, and support the development of personalized vaccination strategies.

2. Materials and methods

This multi-center, multi-national observational case-control study aims to investigate the pathophysiology of TTS/VITT, myocarditis, and

pericarditis following COVID-19 vaccination. Participants are being recruited from INSIS partner sites (Fig. 1).

2.1. Study procedures

INSIS partner sites providing retrospective data identified eligible participants through various means, including clinical referrals, samples submitted for laboratory testing (e.g., PF4 antibody testing for COVID-19 vaccine-associated VITT), and passive and active AEFI surveillance. Eligible participants underwent clinical assessments to confirm the diagnosis and rule out other causes of myocarditis, pericarditis, and TTS/VITT, using diagnostic imaging and other methods as per routine clinical care. Investigations and additional referrals were completed as indicated, according to local and national policies and procedures and physician discretion. INSIS investigators harmonized clinical assessments to the extent possible.

2.2. Ethics statement

All procedures will be performed in compliance with relevant laws and institutional guidelines as approved by the appropriate institutional committees. Informed consent will be obtained prior to study procedures.

2.3. Clinical data collection

All INSIS sites will transfer the raw data from their database to the INSIS REDCap database, which is designed to capture general common data elements for all participants including age, country of enrollment, biological sex, self-reported gender, COVID-19 vaccination details, relevant past medical history, relevant medications, and details of the AEFI/condition including interval from vaccination to symptom onset (if applicable), specific symptoms, and diagnostic results. Details of the AEFIs will be captured in forms derived from the Brighton Collaboration Case Definitions for TTS/VITT and myocarditis/pericarditis to ensure each case is evaluated using consistent criteria. At each follow-up visit, data will be collected on any changed signs or symptoms including those associated with myocarditis, pericarditis, or TTS/VITT to detect persistent or recurrent disease activity.

2.4. Case definitions

2.4.1. Myocarditis/pericarditis

The Brighton Collaboration case definitions ([37], [38]) will be used for both post-vaccine and non-vaccine-associated cases of myocarditis and pericarditis. These events will be categorized into two groups for analysis: myocarditis (with or without signs of pericarditis) and pericarditis without myocarditis. Clinical and demographic features, diagnostic testing results, and multi-omics datasets for these cases will be compared to healthy controls and controls with non-vaccine-associated myocarditis.

2.4.2. Thrombosis with Thrombocytopenia Syndrome (TTS)/Vaccine-Induced Immune Thrombocytopenia and Thrombosis (VITT)

The Brighton Collaboration case definition will be applied to both post-vaccine and non-vaccine-associated cases of TTS/VITT [39,40]. This allows differentiation between vaccine-induced TTS/VITT and non-vaccine associated TTS cases. Clinical and demographic features, diagnostic testing results, and multi-omics datasets will be analyzed for PF4+ VITT cases and TTS not meeting VITT criteria, versus healthy vaccinated controls and controls with non-vaccine-associated TTS/VITT-like conditions [39].

2.4.3. Case-control matching

Cases will be matched to healthy controls, recruited through observational vaccine studies and other approaches, who received the same

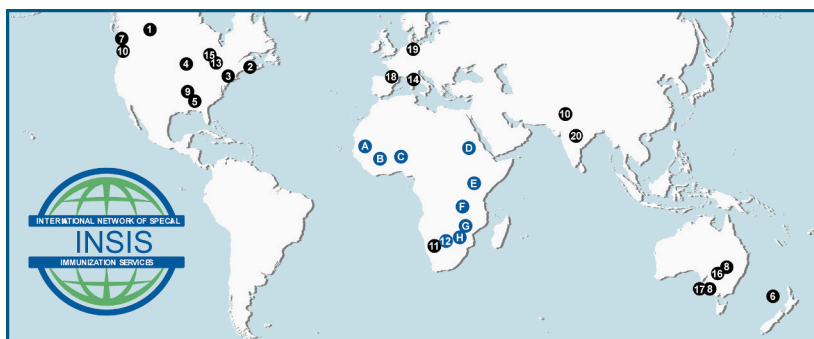


Fig. 1. Map of International Network of Special Immunization Services formal collaborating partners. The participating sites are as follows: ¹INSIS Management Office at the University of Alberta, Edmonton, Alberta, Canada; ²The Hospital for Sick Children (SickKids), Toronto, Ontario, Canada; ³Precision Vaccines Program at Boston Children’s Hospital, Boston, Massachusetts, United States; ⁴Mayo Vaccine Research Group, Rochester, Minnesota, United States; ⁵Brighton Collaboration, Decatur, Georgia, United States; ⁶Global Vaccine Data Network, Auckland, New Zealand; ⁷Canadian Pharmacogenomics Network for Drug Safety, Vancouver, British Columbia, Canada; ⁸Murdoch Children’s Research Institute, Melbourne, Australia; ⁹Vanderbilt Vaccine Research Program, Nashville, Tennessee, United States; ¹⁰University of Washington, Seattle, Washington, United States; ¹¹University of the Witwatersrand, Johannesburg, South Africa; ¹²ALIVE Network (African Leadership in Vaccinology Expertise), Johannesburg, South Africa; ¹³McMaster University, Hamilton, Ontario, Canada; ¹⁴Ospedale Pediatrico Bambino Gesù, Rome, Italy; ¹⁵University of Ottawa Heart Institute, Ottawa, Ontario, Canada; ¹⁶University of Sydney, Sydney, Australia; ¹⁷Monash University, Clayton, Australia; ¹⁸VAC4EU, Brussels, Belgium; ¹⁹University of Southern Denmark; Denmark; ²⁰Global Healthcare Consulting, New Delhi, India; A. Sites in Bamako, Mali; B. Navrongo Health Research Centre, Navrongo, Ghana; C. National site in Nigeria; D. Site in Gondar, Ethiopia; E. Site in Kilifi, Kenya; F. National site in Malawi; G. Maputo City, Mozambique; H. National site in Eswatini.

vaccine platform and, where feasible, the same product (e.g., BNT162b2 or mRNA-1273) and did not develop an AEFI. Samples from these controls will be matched to cases at a ratio of at least 1:1 and ideally 3:1 for multi-omics analysis by age group, biological sex, vaccine type, and ancestry.

Cases may also be frequency matched to controls with a non-vaccine-associated TTS/VITT-like condition (e.g., heparin-induced thrombocytopenia [HIT]) or non-vaccine-associated myocarditis or pericarditis, who meet Brighton criteria and have samples collected and stored at the time of their clinical presentation. Matching will be done by age (± 5 years), biological sex, ancestry (where possible), and vaccine received (for healthy controls).

2.4.4. Sample collection and processing

Both cases and controls will undergo data and sample collection for multi-omics analyses, including transcriptomics, proteomics, and metabolomics using a standardized protocol. Samples will be captured as close to the onset of an AE as possible, up to 12 weeks after the event, with follow-up samples collected as needed. Participants will also be invited to consent to saliva or blood sampling for DNA extraction and genotyping under the GVDN genomics protocol.

Sample collection for cases and controls will be conducted using retrospective residual clinical samples or prospectively at selected sites. At each timepoint (see Table 1), peripheral blood (5–15 ml) will be collected according to protocol. Where processing facilities are available, whole blood (20–30 ml) will be collected for PBMC isolation. Additional sample types, including serum, may be collected according to site-specific or study-specific protocols (refer to INSIS Study SOP in supplementary file 1). Samples for multi-omics analysis will be stored at the participating site until they are ready to be shipped on dry ice or liquid nitrogen to an INSIS laboratory with biobanking facilities, such as the Precision Vaccines Program (PVP), where they will be stored at $-80\text{ }^{\circ}\text{C}$ until analysis (Fig. 2). As of May 15, 2025, 7 sites have entered data for 154 cases and 110 controls into the INSIS REDCap database, with >1100 samples (including serum, plasma, PBMC, and saliva) recorded in the INSIS REDCap database. Additionally, > 1600 sample aliquots have been entered into the Laboratory Data Management System (LDMS) [41] sample management system. These numbers will continue to increase as sites enter their data into the INSIS REDCap and LDMS databases.

Table 1
Schedule of study procedures, prospectively enrolled cases and controls for multi-OMICs analysis. *†, ‡

	During adverse event/onset myocarditis/VITT/TTS	2–8 weeks after event onset†	~12–20 weeks after onset	~6–24 months after onset
Cases and controls with non-vaccine associated disease	<ul style="list-style-type: none"> • Consent (if possible) • Data collection • Blood collection* 	<ul style="list-style-type: none"> • Consent† • Data collection • Blood collection 	<ul style="list-style-type: none"> • Consent (if not yet obtained) ‡ • Data collection • Blood collection 	<ul style="list-style-type: none"> • Data collection • Blood collection
Controls	<ul style="list-style-type: none"> • Consent (if possible) • Data collection • Blood collection** 	<ul style="list-style-type: none"> • ~3–90 days post-vaccination** • Consent† • Data collection • Blood collection 	<ul style="list-style-type: none"> • ~12–20 weeks post-vaccination • Consent (if not yet obtained) ‡ • Data collection • Blood collection 	<ul style="list-style-type: none"> • ~6–24 months post-vaccination • Data collection • Blood collection

* Participants may be included if multi-OMICs samples are available from only 1 timepoint but 2 or more timepoints are preferred within these approximate timeframes (preferably an acute sample and follow up sample). In some cases an early follow up sample and late follow up sample (e.g., 3 and > 6 months) will be acceptable. A maximum of 90 ml of blood will be drawn on children <16 years of age.

† At time of first specialist assessment (e.g., cardiology, special immunization clinic).

‡ If not consented at presentation, consent will be obtained at time of specialist assessment for retrieval of residual serum/plasma from initial presentation.

** Aim for 3–7 days post-vaccination for controls matched to myocarditis cases; 5–42 days post-vaccination for controls matched to VITT cases, longer timeframes may be appropriate for additional AEFI of interest.

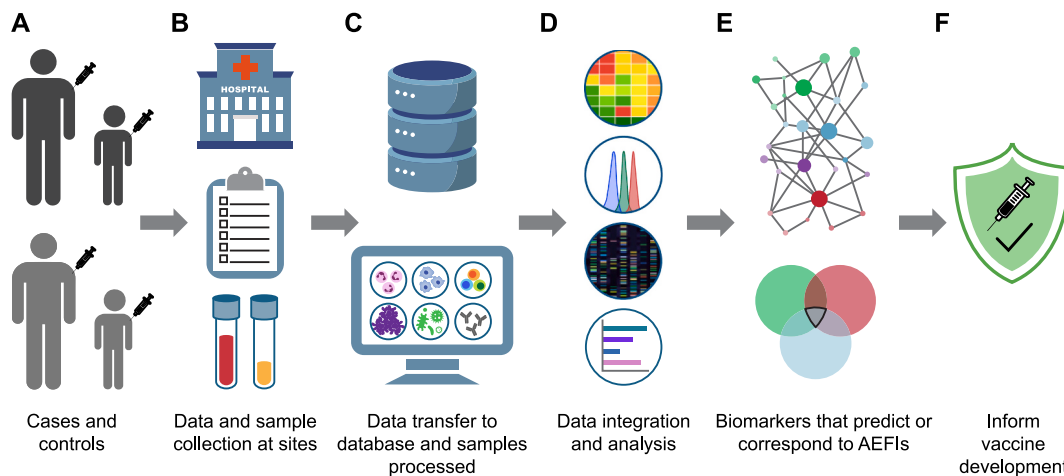


Fig. 2. The International Network of Special Immunization Services (INSIS) approach to defining biomarkers of vaccine-associated adverse events. A) AEFI cases and controls are recruited. B) Cases and controls undergo standard assessment, data and sample are collected at INSIS clinical assessment centers. C) Data are transferred to the INSIS central database, and samples are processed at INSIS laboratories for multi-OMICs. D) Integration and analysis of clinical and biological data. E) Biomarkers that predict or correspond to AEFIs will be identified. F) Results will inform vaccine development and personalized vaccination strategies. Abbreviation: AEFI, adverse event following immunization.

2.4.5. Core laboratory assays and technologies

The following laboratory analyses will be performed on samples collected for this study using a downstream sample-sparing technique for systems biology analyses. Fig. 3 outlines the assay prioritization workflow for the INSIS project (Fig. 3).

2.5. Proteomics

2.5.1. Untargeted mass spectrometric profiling

Proteomics will be performed (Steen Lab, Boston Children's Hospital; Boston, MA) employing a two-pronged approach [42]. First, the plasma proteome will be quantitatively mapped without any depletion considering the important immunomodulatory roles of a sizable fraction of the standard depletion targets such as immunoglobulins and complement pathway components [43,44]. Mapping the vaccine and AEFI-associated changes in abundance for these proteins is important for understanding the pathophysiological processes of these AEFIs. To map the 'classical' plasma proteome, the neat plasma will be processed in a high throughput fashion. To map the 'tissue leakage' plasma proteome, we will deplete the most abundant plasma samples using perchloric acid, which can be conducted in a high-throughput and cost-efficient manner on thousands of samples [45–47].

The two resulting plasma protein digests will be analyzed by LC-MS in discovery mode using a high-throughput sample delivery and high-performance liquid chromatography (HPLC) system (Evosep One) front-end and a Bruker ion mobility/quadrupole/TOF mass spectrometer (timsTOF HT) back-end to ensure robustness. The instrument will be operated in Data Independent Acquisition (DIA) mode to ensure maximum completeness of the data. All data will be qualitatively and quantitatively analyzed using the Bruker ProteoScape hard- and software environment.

2.5.2. Proximity extension assay (PEA) proteomics (Olink)

Targeted proteomics using Olink's Proximity Extension Assay (PEA) will be applied to plasma or serum employing a core lab certified workflow. This approach allows for the simultaneous investigation of 92 proteins in 88 samples offering the flexibility to choose from distinct panels (kit target 96, T96) or 45 proteins in 40 samples (kit target 48, T48), using just 1 μ l of sample. This technology has also been validated in other biomatrices such as supernatants, serum, tears, biopsies and depending on the kit used, it provides relative (NPX values) or absolute quantitative (pg/ml) results, respectively, in T96 and T48. The kits can

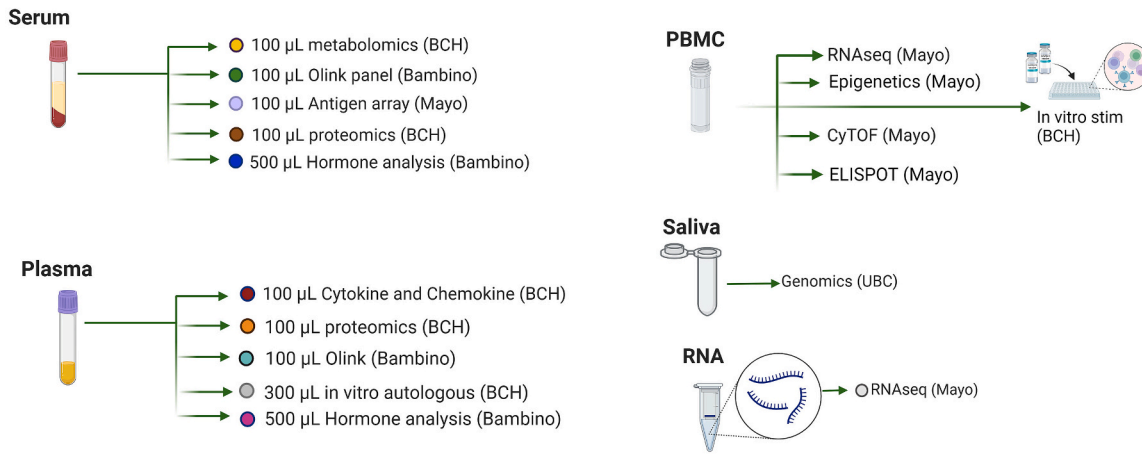
also be customized to focus on a specific pathogen/immunological question. This approach will provide additional insights into the protein signatures characterizing AEFIs, comparing them with other conditions, including healthy controls, as previously described in smaller cohorts [48].

2.5.3. Untargeted metabolomics profiling

Metabolomics offers a powerful tool for understanding vaccine safety events by providing comprehensive insights into the biochemical changes associated with vaccination. By detecting specific and consistent metabolic signatures, metabolomics allows for the precise identification of disease states or vaccine responses [49]. For example, alterations in prostaglandin metabolites and eicosanoids, related to hyperinflammation, may help pinpoint the biochemical basis of adverse reactions [50]. Additionally, metabolic dysregulation has been observed in COVID-19 hospitalized patients with severe disease trajectories, including decreased phospholipid components and elevated plasma branched-chain amino acid (BCAA) and urea components captured by untargeted metabolomics [51]. Furthermore, integrating metabolomics with other systems biology platforms can provide a holistic view of the host immune response [49].

Plasma metabolomics assay for the INSIS project will be conducted (Metabolon, Durham, NC) using a previously described workflow [49]. Samples will be randomized into batches, extracted, and prepared for analysis using solvent extraction method [52]. Recovery standards will be added at the initial extraction step to ensure quality control. Proteins will be precipitated with methanol under vigorous shaking and then centrifuged. The resulting supernatants will be divided into five fractions for various analyses, including two reverse phase (RP)/UPLC-MS/MS methods with positive ion mode electrospray ionization (ESI), one RP/UPLC-MS/MS with negative ion mode ESI, one HILIC/UPLC-MS/MS with negative ion mode ESI, and one reserved for backup analysis using high-resolution mass spectrometry. Metabolites will be identified by comparing results to a library of standard metabolites [52] using criteria such as retention index, accurate mass match, and MS/MS scores. Compounds will be categorized according to standards set by the Metabolomics Standards Initiative [53–55]. Appropriate analytical techniques will be used to validate and report metabolites of interest, ensuring accurate and reliable data for further analysis.

A INSIS Retrospective Samples



B INSIS Prospective Samples

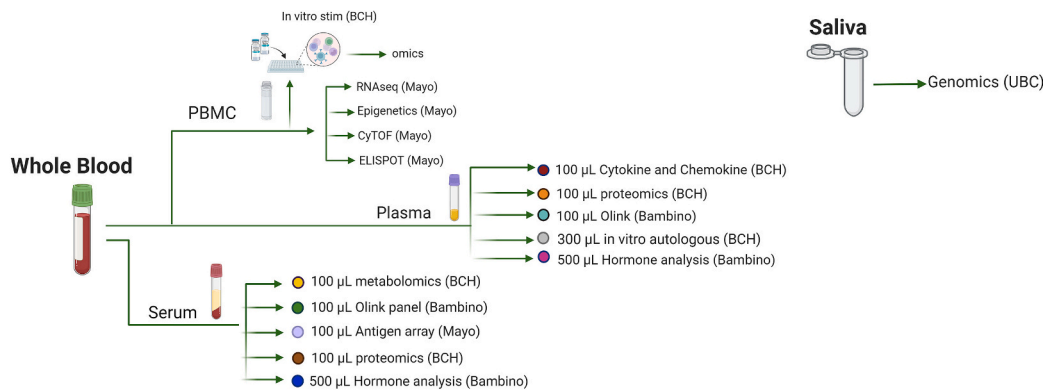


Fig. 3. INSIS Sample Processing Pipeline and Core Lab assays. A) The following assay prioritization will be used for retrospective samples: Serum samples will be used to measure metabolomics, proximity extension assay proteomics (Olink), antigen array, proteomics, and hormone analysis. Plasma will be used to measure cytokines and chemokines, proteomics, Olink, human in vitro modeling and hormone analysis. PBMCs will be used for RNAseq or transcriptomics analysis, epigenetics, human in vitro modeling, immunophenotyping using mass cytometry time of flight (CyTOF) and ELISPOT. Saliva will be used for genomics and RNA for transcriptomics analysis. B) For prospective samples; whole blood collected in tubes with an anticoagulant (EDTA preferably) will be processed for PBMCs and plasma samples. Serum will be collected in tubes without any anticoagulant (e.g. SST Greiner tubes). Saliva will be used for genomics. The retrospective samples processing pipeline will be followed for all prospective sample types. Core Labs: Boston Children’s Hospital (BCH), Clinical and Research Unit of Clinical Immunology and Vaccinology, Bambino Gesù Children’s Hospital (Bambino), Mayo Clinic College of Medicine (Mayo), University of British Columbia (UBC).

2.6. Hormone analysis

Considering the higher incidence of myocarditis in males post SARS-CoV-2 infection and vaccination, it has been suggested that androgens may play a potential role in post-COVID-19 vaccination cardiac events [56]. Furthermore, extensive research has been conducted on the impact of androgens on the immune system [57].

Steroid hormone measurement will be conducted on suitable plasma and/or serum samples using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Such analysis will be conducted (Clinical Biochemistry Laboratory, Department of Diagnostic Medicine of the I.R.C.C.S. Bambino Gesù Children’s Hospital) using the Xevo TQ-Smicro Mass Spectrometer of Waters. Sample preparation will be carried out using the CE-IVD certified diagnostic kit of Chromsystems (Munich, Germany) (or equivalent), and samples will be processed on an appropriate platform such as a mass spectrometer equipped with an ACQUITY UPLC I-Class liquid chromatograph that allows the realization of an ultra-high performance and low dispersion liquid chromatography optimized to derive maximum benefits in terms of resolution and sensitivity, and a triple quadrupole that complies with Directive 98/79/

EC in all its parts. This type of instrumentation couples ultra-high-performance, low-dispersion chromatography with triple quadrupole used in Multiple Reaction Monitoring (MRM) mode, a specific method developed to detect specific peptides in complex biological mixtures such as human plasma and serum.

2.7. SARS-CoV-2 antigen array

Vaccine-associated AEs may have an immunologic trigger. We will use a pathogen proteome array to screen the humoral immune response to SARS-CoV-2 and other relevant coronaviruses in cases and controls to identify differences in humoral immune response generated in those who do and do not experience our selected AEs. Antibody responses to the SARS-CoV-2 proteome will be characterized using a commercial multi-coronavirus protein microarray (Antigen Discovery Inc.; Irvine, CA, USA). The array includes 935 full-length proteins, overlapping protein fragments and overlapping 13–20 aa long peptides from SARS-CoV-2 (WA-1), SARS-CoV, Middle East respiratory syndrome coronavirus (MERS-CoV), human coronavirus (HCoV)-NL63, and HCoV-OC43. Proteins will be expressed using an *Escherichia coli* in vitro

transcription and translation (IVTT) system (Rapid Translation System, Biotech Rabbit, Berlin, Germany). Sera samples will be diluted in PBS, incubated on the microarray. Following removal of the residual serum, bound antibodies are detected using fluorochrome-labelled, anti-human Ig reagents [58–60].

2.8. Cytokine and chemokine secretion analysis

Cytokines and chemokines, low-molecular-weight proteins, play crucial roles in the immune response to infection and vaccination. Elevated plasma cytokine levels are associated with severe COVID-19, while vaccination appears to reduce inflammation, potentially mitigating disease severity and mortality. Aberrant cytokine production may contribute to the pathophysiology of severe COVID-19 [61] [62] as well as the vaccine-related AEs [63–65].

The multiplex cytokine and chemokine assay procedure has been previously described [66] and uses the Milliplex Human Cytokine/Chemokine Magnetic Bead Premixed 41 Plex Kit. (cat. #HCYTMA60 K-PX41). Cytokines and chemokines measured using the 41-plex Milliplex Map Kit contains; sCD40L, EGF, FGF-2, Flt-3 ligand, Fractalkine, G-CSF, GM-CSF, GRO (CXCL1), IFN- α 2, IFN- γ , IL-1 α , IL-1 β , IL-1ra, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, CXCL8, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17 A, IP-10 (CXCL10), MCP-1 (CCL2), MCP-3 (CCL7), MDC (CCL22), MIP-1 α (CCL3), MIP-1 β (CCL4), PDGF-AB/BB, RANTES (CCL5), TGF- α , TNF- α , TNF- β , VEGF, Eotaxin (CCL11), and PDGF-AA. Adult plasma samples will be assayed undiluted. The samples will be assayed using a 384-well plate (Corning CellBIND® (cat. #CLS3764)) platform following the manufacturer's instructions, including the standards and quality controls provided by the kit. Samples will be run and fluorescent signals will be acquired using a Flexmap 3D system with Luminex xPONENT software (Luminex Corp.; Austin, TX, USA). 5-parameter logistic, and exponential functions will be used to fit to the dilution series data per analyte, selecting the best fit function in each case, using Milliplex Analyst Software version 5.1. The curves will be used to determine the lower and upper limits of detection and quantification for each analyte and plate. Analytes that fall below or above these values will be imputed to the lower or upper limit of quantification, respectively. For any given sample and analyte, concentration values will be discarded if readings are <30 beads. Samples with all analytes below the lower limit of detection will be excluded from analysis.

2.9. RNA sequencing

AEIs may be caused by overactive or inappropriate immune responses targeting self-antigens, adverse reactions to viral protein expression, or other dysregulated physiologic responses. These responses must begin with transcriptional changes in the reactive cells and/or tissues. Gene expression analysis of PBMCs will be used to evaluate whether there are transcriptomic signatures associated with AEs following COVID-19 vaccination that can be detected in the peripheral blood. In the case of VITT/TTS, the primary pathology is blood-based and therefore PBMC transcriptomic data will complement the additional analysis of the proteome and metabolome in blood, providing a fuller picture of the molecular mechanisms underlying this adverse event.

PBMC samples will be thawed and cultured under appropriate conditions (e.g., unstimulated or antigen-stimulated) at the Mayo Clinic Vaccine Research Group (MVRG) [67]. RNA will be extracted using Qiagen kits and sequencing libraries will be prepared using the TruSeq Stranded mRNA Library Prep kit. mRNA-Seq will be performed in the Mayo Clinic Medical Genome Facility using the Illumina NovaSeq 6000 platform. Flow cell samples will be sequenced as 51 × 2 paired-end reads using HCS v2.0.12 data-collection software or an equivalent platform. Base-calling will be performed using Illumina's RTA version 1.17.21.3. Gene sequencing data will be aligned using the MAP-Seq V1 pipeline to

the h19 human genome.

2.10. Immunophenotyping using mass cytometry time of flight

Immunopathology can be mediated by inappropriate antigen stimulation of lymphocyte populations (e.g., self-reactive T cells) or activation of innate immune cells (e.g., mast cells or basophils triggering anaphylaxis) [68]. Immunophenotyping panels provide an opportunity to comprehensively evaluate both activation status and expansion/contraction of critical immune cell populations that may be responsible for vaccine-related SAEs [69]. CyTOF will be performed with the use of mass cytometry through Mayo Clinic's Immune Monitoring Core. The Core has a validated panel consisting of 36 antibody markers covering a broad range of leukocyte populations (supplementary file 2) [70].

2.11. Genomics analyses

2.11.1. Genome-wide association studies (GWAS)

Extracted DNA from saliva samples will be genotyped with a custom Illumina Global Screening Array (GSA version 3.0 with additional pharmacogenomic content) including genetic variation throughout the genome (500,000 genome-wide markers), further enriched with pharmacogenomic variants including >45,000 variants in core drug absorption, distribution, metabolism, and elimination genes, and > 24,000 variants in major histocompatibility complex (MHC)/HLA gene regions. The array captures both common and rare variants collected from large-scale sequencing projects.

Previously reported candidate genes potentially related to the pathogenesis or biological mechanisms of GBS, TTS/VITT, myocarditis, and pericarditis will be also genotyped by either the custom GSA array or by custom TaqMan genotyping assays. Candidate genes for GBS include, but are not limited to, HLA alleles, IL-10, KIR, TNF- α , CD1, and Fc γ R. Candidate genes for TTS/VITT include, but are not limited to, F5, F2, PROC, and PROS1. Candidate genes for myocarditis and pericarditis include, but are not limited to, BAG3, DSP, PKP2, RYR2, SCN5A, and TNNT1 [71,72].

Genotyping will be followed by whole genome imputation of common variants using SHAPEIT (v2) and IMPUTE2 (v2.3.2) in combination with the Phase 31,000 Genomes Project reference panel and imputation of classical HLA alleles and HLA-region variants using SNP2HLA (v1.0.2) in combination with Type 1 Diabetes Genetics Consortium (T1DGC) reference panel. This will yield a final genotyped and imputed dataset of ~10 million variants per sample.

2.11.2. Exome sequencing

From each of the three case groups, 50 of each the most severe AE patients who are categorized as Brighton Collaboration Level One cases of COVID-19-induced GBS, TTS/VITT, or myocarditis/pericarditis will also be selected (a total is 150 for three AEs) to perform exome analyses. This will complement genome-wide genotyping, particularly in protein-coding regions, to identify the most possible disease-causing mutations. The public exome sequencing database, gnomAD, as reference controls will be used to investigate novel and rare genetic variants related to these three specific AEs. Following library preparation with an IDT Capture Expanded Exome Kit, exome sequencing to a mean coverage of 100× will be performed using paired end sequencing (2 × 150 bp) on an Illumina Sequencing platform (NovaSeq platforms). The sequence data will be processed according to GATK Best Practices (v4), using BWA-MEM for alignment of reads to the GRCh38 reference genome on the local high-performance computing cluster. Significant variants identified from the GWAS discoveries will be further validated by genotyping (e.g., TaqMan assays) or sequencing.

2.12. Epigenetics

Gene expression is controlled, in part, by epigenetic regulatory

features such as DNA methylation. The aim of these experiments is to perform an unbiased measurement of DNA methylation patterns across CpG sites across the genome. DNA methylation patterns that do correlate with vaccine SAEs may provide insights into the biological activities that are dysregulated and contribute to those AEs. Following DNA extraction from PBMC samples, genome-wide methylation patterns will be assessed using either an Illumina Methylation BeadChip or RBBS followed by methyl-Seq.

2.13. Human in vitro modeling

To assess whether vaccine-induced molecular signatures may predict AEFIs, where facilities exist, we will collect study participant blood to generate cryopreserved PBMCs and matched autologous plasma. These will be batch shipped to the Precision Vaccines Program (Boston Children's Hospital, Boston, MA) to conduct human in vitro assays such as PBMCs cultured in 10 % autologous plasma and tissue construct assays as previously described [73–75]. Conditions tested will include vehicle control, agonists of pattern recognition receptors (PRRs) involved in COVID-19 vaccine-induced innate immune responses [76], and authorized/approved COVID-19 vaccines at three dilutions: 1:1000, 1:100 and 1:10 vol/vol. Cellular and soluble fractions will be collected and cryopreserved for downstream systems biology as we have described [77]. Resulting cellular and molecular signatures will be integrated with clinical and immunologic data to provide insights into modeling human in vitro vaccine responses in relation to AEFIs, an approach supported by the United States Food and Drug Administration (FDA) Modernization Act 2.0 [78] aimed at identifying actionable biomarkers to inform future vaccine discovery and development. This human in vitro modeling system provides an ability to recapitulate key aspects of tissue-specific immune responses [70,75,79,80] adding important mechanistic insight to complement in vivo analyses.

3. Data management and analytical strategy

3.1. Study oversight and reporting

Data are submitted to a central REDCap database hosted and maintained by the Data Management and Analysis Core (DMAC) (PVP, Boston Children's Hospital; Boston, MA, USA). Participant data will be transferred to the INSIS REDCap database from participating INSIS networks and partners using secure transfer protocols. INSIS sites will be responsible for local quality control and retain ownership of their data. The DMAC will conduct additional data quality checks on the database and maintain a centralized data repository and analytic platform.

Standard forms and labels will be used for sample tracking and linkage to clinical databases. Patient samples will be linked to their record in the database by a unique study ID. The sample management system LDMS [41] will be used to track samples from the point of collection to the analytic laboratories by scanning a sample-specific barcode, which carries a unique sample identifier (sample ID). Date of sample collection, sample type, and volume at each timepoint will be recorded on a sample processing form (SPF) and these metadata will be captured electronically by the central INSIS database. The DMAC will oversee sample tracking and shipping.

Within the framework of executed material transfer and data transfer use agreements (MTAs and DTUAs), and appropriate institutional approvals, INSIS sites will partner with the PVP, Mayo Vaccine Research Group MVRG, Ospedale Pediatrico Bambino Gesù (OPBG), and other INSIS affiliated laboratories to coordinate sample shipping for the multi-omics analysis described above. Integration of clinical and biological data will be conducted using a cloud-based bioinformatic analytic infrastructure. INSIS will work with GVDN to coordinate transfer of clinical data and DNA samples collected at INSIS sites to GVDN (Canadian Pharmacogenomics Network for Drug Safety (CPNDS) lab at University of British Columbia) for processing and analysis.

3.2. Data harmonization framework

Harmonized procedural and data processing pipelines across assays are coordinated via the INSIS Data Management Working Group (DMWG) with support from the DMAC. As the cohort is recruited, plans will address missing clinical data and samples and appropriately randomize samples for each assay type to eliminate selection bias and evenly distribute confounding variables. Power calculations will be conducted to ensure that the study is adequately powered to detect significant differences or associations in the data, accounting for potential variability and expected effect sizes. Standardized metadata templates and controlled vocabularies will be applied to ensure data interoperability across sites and assay platforms. As part of our quality control and assurance measures, we recognize that self-reported demographic variables such as gender and race may vary in consistency across study sites. We will incorporate both self-reported and where available, biologically inferred variables (e.g. genetic ancestry, sex chromosome-linked gene expression) during analysis.

Once the data are uploaded to the INSIS study database, DMAC data managers and biostatisticians will coordinate and verify quality control (QC) processes for data collected/generated at clinical sites and Core Labs. They will also perform additional quality assurance (QA) to maintain the highest possible accuracy of clinical, immunologic, and systems biology data before reporting and analysis in the centralized cloud computing system (Fig. 4).

3.3. Centralized computational platform and data deposition

A cloud computing platform specifically for the INSIS study will be utilized for encrypted, access-controlled data storage and analysis resources [81]. This will include a data and analysis dashboard that allows INSIS investigators to upload, store and analyze both raw and processed computable data within a centralized computing environment. The computing platform will offer a secure environment for developing, testing, and running scripts, as well as performing quality control (QC) and quality assurance (QA) on data generated by the Core Labs. This centralized system will ensure that the INSIS Core Labs adhere to shared data standards and maintain internal consistency, facilitating accurate and integrated data analysis. This setup will be designed to facilitate data sharing and downstream analyses by INSIS investigators and the broader research community, who will be able to access the data and associated metadata via a public data repository such as dbGAP [82] or ImmPort [83] (immport.niaid.nih.gov). Deidentified quality assured published data will be deposited to public repositories according to the funder's policies.

3.4. Analysis of clinical features and outcomes

Clinical features, results of investigations, other exposures or risk factors, and outcomes of the AEFI will be compared within each subgroup by vaccine product, age, biological sex, self-reported gender, and race. Examples of analysis will include multivariable regression analysis which will identify demographic and clinical factors associated with Brighton Collaboration case definition-confirmed TTS/VITT, myocarditis, and pericarditis in cases versus healthy controls. Additionally, we plan to compare factors related to post-vaccination versus non-vaccine associated myocarditis and TTS/VITT versus HIT/VITT-like non-vaccine associated syndromes. Similar approaches will be employed for new AEFIs that emerge as safety signals.

With sufficient sample size, some samples may be used for discovery cohorts and others for replication, with one network (e.g., INSIS) leading an omics discovery analysis and providing samples for the replication of another network's analyses. Similar sample size and matching approaches will be used for new AEFI targets. INSIS will partner with GVDN to recruit cases and controls for genomics analysis in GVDN-led studies.

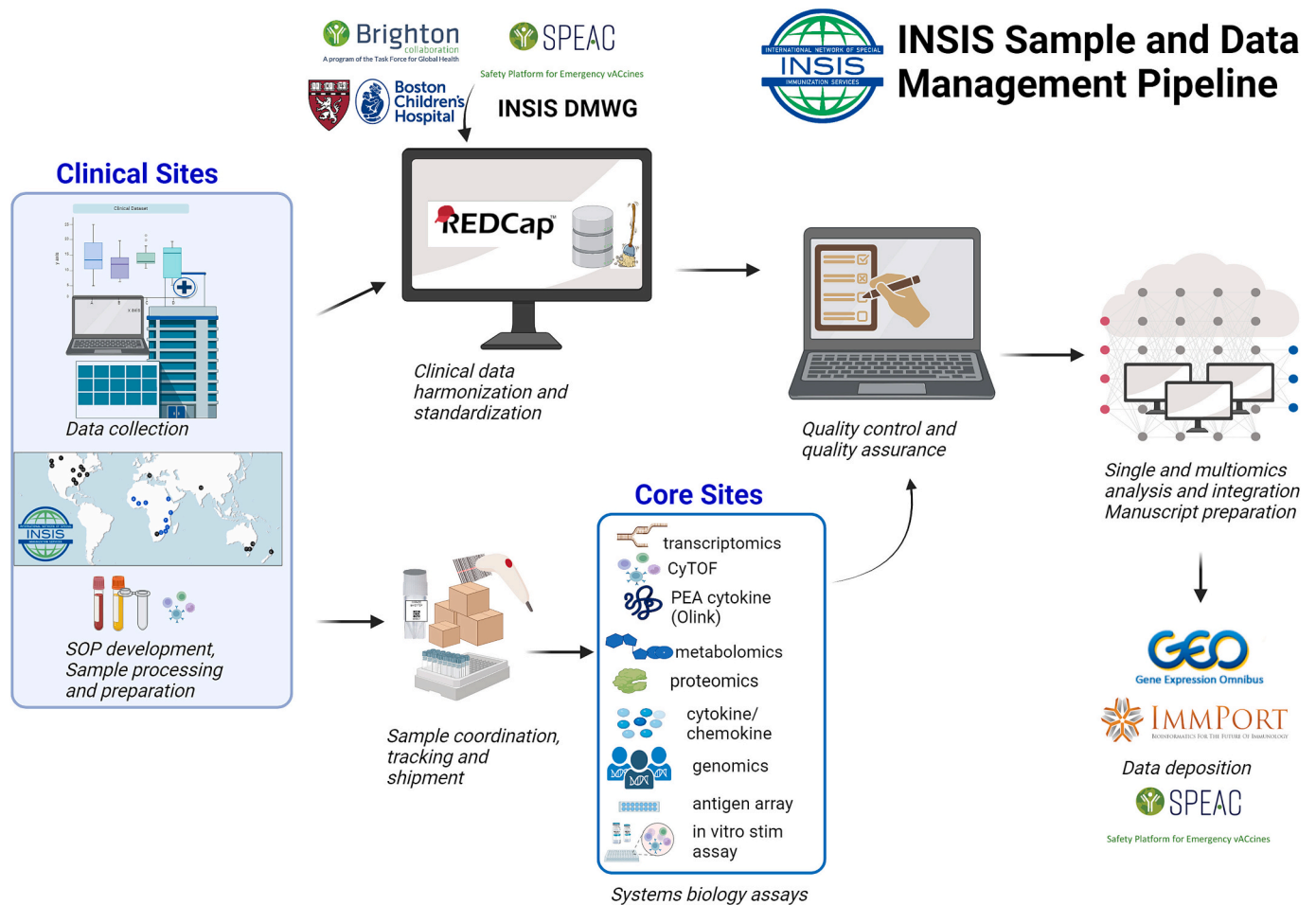


Fig. 4. INSIS Sample and Data Management Pipeline. This figure illustrates the comprehensive workflow for sample and data management within the INSIS study. Data collection is performed at various clinical sites, involving the development of standard operating procedures (SOPs), as well as sample processing and preparation. The map illustrates the global locations of these clinical sites. Collected clinical data are harmonized and standardized by the Data Management Working Group (DMWG) using the REDCap platform, in collaboration with the Brighton Collaboration and the Safety Platform for Emergency Vaccines (SPEAC). A rigorous quality control (QC) and quality assurance (QA) process ensures the accuracy and reliability of both data and samples before analysis. Specialized core sites conduct various assays and omics analyses, including transcriptomics, Cytometry by Time-Of-Flight (CyTOF), Proximity Extension Assay (PEA) for cytokine profiling (Olink), metabolomics, proteomics, cytokine/chemokine analysis, genomics, antigen array analysis, and in vitro stimulation assays. The pipeline includes meticulous sample coordination, tracking, and shipment to ensure samples are handled and processed correctly. Single and multi-omics data are analyzed and integrated within a centralized computing environment, facilitating comprehensive data interpretation and manuscript preparation. Final datasets and associated metadata are deposited in public repositories such as NCBI Gene Expression Omnibus (GEO) and ImmPort, ensuring data accessibility for the broader research community.

3.5. Integrated multi-omics analysis

Multi-omics data from patients with well-defined phenotypes (e.g., Brighton level 1 TTS/VITT) and controls will be analyzed using data integration approaches such as MultiOmics Factors Analysis (MOFA) and Data Integration Analysis for Biomarker Discovery using Latent Components (DIABLO) as previously described [84,85]. MOFA is a computational method used to integrate and analyze multi-omics datasets which distinguishes between patterns that are shared across different omics layers and those that are specific to individual layers [84]. DIABLO identifies key drivers associated with the response variable of interest across all input data matrices jointly. Cross-validation will determine the optimal model hyperparameters (number of components, features per component) and estimate the model's ability to generalize to new data. Selected model features will undergo pathway over-representation analysis against the Reactome pathways database (via MSigDB) and blood transcriptional module (BTM) annotated gene set libraries, with *p*-values adjusted to control false discovery rate (FDR).

Multi-omics data will be compared within groups over time (e.g., myocarditis/TTS/VITT onset vs post-recovery) and between cases and

controls to identify differences in basal levels of analytes in cases (using baseline samples: pre-vaccination, post-recovery/ ≥ 3 months post-vaccination), as well as at time of myocarditis/TTS/VITT diagnosis (and similar timepoints post-vaccination in controls). Analyte levels in blood/plasma will also be compared to normal ranges in adults where available. Two-way comparisons will be performed between cases and healthy controls vs cases and controls with non-vaccine associated myocarditis or HIT.

4. Governance and organizational structure

INSIS is a global consortium focused on AEFIs and involving key clinical consultation services including the SIC Network, AEFI-CAN, members of the US Clinical Immunization Safety Assessment (CISA) network, Brighton Collaboration, GVDN, African Leadership in Vaccinology Expertise (ALIVE) network, experts in systems immunology (PVP, Boston Children's Hospital; MVRG, OPBG, Rome, IT) and pharmacogenomics (University of British Columbia and BC Children's Hospital Research Institute), and experts in pharmacogenomics and global vaccine policy (Global Healthcare Consulting and University of

Washington). INSIS is managed by the Network Management Office (NMO) at the University of Alberta, under the leadership of the Nominated Principal Investigator (NPI) and Brighton Co-lead. The NMO coordinates operations, including finance, contracts, and communications, and interacts with the Task Force for Global Health, which hosts the Brighton Collaboration.

The Steering Committee, comprising representatives from key clinical networks and experts in vaccine safety and systems biology, oversees network governance, project progress, and funding. Monthly meetings facilitated by the NMO ensure alignment with project milestones. Specialized Working Groups, such as Data Management and OMICs, also meet monthly to support project deliverables. INSIS holds monthly calls open to all members to discuss and plan project progress and feature guest speakers on vaccine safety topics.

4.1. Scientific advisory board and dissemination strategy

INSIS will establish a Scientific Advisory Board (SAB) with diverse representation from funders, regulators, public health experts, and stakeholders, particularly from LMICs. The SAB will provide guidance on research priorities and ensure results are aligned with stakeholder needs and translated into policy.

INSIS will disseminate findings through its website, reports, presentations, open-access publications, and conferences. A Publication and Presentation Policy, following ICMJE guidelines, governs authorship and dissemination. Results will also be shared with participants and the public through institutional websites, media channels, and social media, with support from partner organizations' communications teams.

5. Conclusions

The establishment of INSIS marks a significant advancement in the global effort to understand and mitigate rare AEFIs associated with COVID-19 vaccination. By integrating clinical data with advanced multi-omics technologies, INSIS provides a comprehensive platform for investigating the underlying mechanisms of AEFIs such as COVID vaccine-induced immune thrombocytopenia and thrombosis (VITT) and myocarditis. The rigorous data management and quality assurance processes employed by INSIS will ensure the accuracy and reliability of the collected data, facilitating robust analyses and meaningful conclusions. This approach will not only enhance our understanding of these conditions but also will inform vaccine development and development of personalized vaccination strategies, ultimately aiming to improve public health outcomes.

As the work progresses and the network continues to expand, INSIS aims to apply the methodology described herein to new AEFIs and additional vaccines targeting diverse threats. INSIS' collaborative framework and cutting-edge methodologies will remain crucial in addressing emerging vaccine safety signals. INSIS' efforts highlight the importance of global collaboration in vaccine safety research and emphasize the potential of integrating clinical, immunologic, and multi-omics analysis to drive scientific discoveries and inform public health policies.

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Declaration of competing interest

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.vaccine.2025.127504>.

Data availability

No data are included in this paper, as it is a study protocol. This document outlines the planned methodology and study design. Any data that will be generated during the course of the research will be shared in accordance with our study protocol guidelines for data deposition and will be accessible upon reasonable request. The data will be deposited in a public repository once the study is completed and all necessary ethical approvals are obtained.

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