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Changes in maternal immunity during pregnancy can result in an altered immune state and, as a natural perturbation, this provides an opportunity to understand functional interactions of the immune system in vivo. We report characterisation of maternal peripheral immune phenotypes for 33 longitudinally sampled normal pregnancies, using clinical measurements of complete blood counts and major immune cell populations, as well as high parameter flow cytometry for 30 different leukocyte antigens characterising 79 cell populations, and monitoring of 1305 serum proteins using the SomaLogic platform. Cellular analyses characterised transient changes in T cell polarization, and more persistent alterations in T and B cell subset frequencies and activation. Serum proteomic analysis identified a novel set of 7 proteins that are predictive of gestational age: DDR1, PLAU, MRC1, ACP5, ROBO2, IGF2R, and GNS. We further show that gestational age can be predicted from the parameters obtained by complete blood count tests and clinical flow cytometry characterizing 5 major immune cell populations. Inferring gestational age from this routine clinical phenotyping data could be useful in resource limited settings which lack obstetric ultrasound. Overall, both the cellular and proteomic analyses validate previously reported phenotypic immunological changes of pregnancy, and uncover new alternations and predictive markers.

Title: Multi-modal immune phenotyping of maternal peripheral blood in normal human pregnancy

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Abstract

Changes in maternal immunity during pregnancy can result in an altered immune state and, as a natural perturbation, this provides an opportunity to understand functional interactions of the immune system in vivo. We report characterisation of maternal peripheral immune phenotypes for 33 longitudinally sampled normal pregnancies, using clinical measurements of complete blood counts and major immune cell populations, as well as high parameter flow cytometry for 30 different leukocyte antigens characterising 79 cell populations, and monitoring of 1305 serum proteins using the SomaLogic platform. Cellular analyses characterised transient changes in T cell polarization, and more persistent alterations in T and B cell subset frequencies and activation. Serum proteomic analysis identified a novel set of 7 proteins that are predictive of gestational age: DDR1, PLAU, MRC1, ACP5, ROBO2, IGF2R, and GNS. We further show that gestational age can be predicted from the parameters obtained by complete blood count tests and clinical flow cytometry characterizing 5 major immune cell populations. Inferring gestational age from this routine clinical phenotyping data could be useful in resource limited settings which lack obstetric ultrasound. Overall, both the cellular and proteomic analyses validate previously reported phenotypic immunological changes of pregnancy, and uncover new alternations and predictive markers.

Introduction

Epidemiological observations show that maternal immunity is perturbed during normal human pregnancy. Increased risk of severe disease is reported in pregnancy for defined infections including listeriosis, malaria and varicella (1-3). Worse outcomes of influenza infection, and diminished antibody responses to influenza vaccination, are observed in pregnant women (4, 5). On the other hand, specific maternal autoimmune disorders such as rheumatoid arthritis and multiple sclerosis may regress during pregnancy, whereas symptoms of systemic lupus erythematosus and myasthemia gravis may worsen (6-8). The immunological changes during pregnancy which underlie these effects are complex and remain poorly understood. Immunological accommodation of the semi-allogeneic foetus is mediated primarily by the dedicated tissues of maternal decidua and fetal placenta, which exhibit multiple features of opposing selective forces in viviparity including imprinting (9). Maternal peripheral immunity is also clearly altered, with changes in systemic IL-4 levels and frequencies of T_H2 cells described in normal pregnancy over 25 years ago (10, 11). Subsequent work has continued to define the changes in peripheral immune phenotypes, to add to our understanding of the physiologically significant modifications of maternal immunity that occur during normal pregnancy (12, 13). This understanding could potentially address the clinical susceptibilities or resistance to some pathologies during pregnancy, or help the design of maternal immunization.

Given the broad nature of peripheral maternal immunological changes during pregnancy, high dimensional phenotyping is particularly appropriate. Systems biology analyses of intracellular signalling responses measured by mass cytometry, and combined with other modalities, have been used to define molecular clocks of gestational age (GA) (14, 15). In addition to biological understanding of the changes during gestation, these clocks could be used to detect pathologies of pregnancy such as preterm birth. To complement these data, we have conducted a multi-modal high dimensional analysis of longitudinally sampled individuals in the context of normal

pregnancies. Here, we identified changes in maternal peripheral immunity, focussing on immune cell populations and the serum proteome. This analysis allowed us to validate previous findings, identify novel changes throughout pregnancy, and determine models predictive of GA that use minimal sets of serum proteins or routinely acquired clinical phenotyping data.

Results

Study design

For 33 pregnancies with vaginal delivery of healthy singletons, 4 peripheral blood samples were obtained from 3 timepoints throughout gestation as well as once after delivery. The distribution of sampling timepoints and cohort characteristics are summarized in Fig. 1 and Table 1. For all 132 samples immune cell phenotypes were characterized by high parameter flow cytometry, and the serum proteome was analysed using the SomaLogic platform. Measurements were also obtained from complete blood count (CBC) tests, and low parameter clinical flow cytometry quantifying 5 major immune cell populations.

Immune cell population phenotypes

Peripheral blood immune cell phenotypes were studied by high-parameter flow cytometry to quantify 79 cellular subsets (Suppl Tab. 1 and 2). Populations that significantly changed in frequency were identified by longitudinal paired tests comparing between visit 1 (early gestation), visit 3 (late gestation) or visit 4 (post-partum). We detected 32 population subsets that differed significantly in at least one of these comparisons (fold change>1.2 or <0.8, q<0.05) (Fig. 2A). In some cases, the same population is identified by more than one of the parallel staining panels used, and technical validation is provided by detection of similar changes in these populations. An example is the increase in HLA-DR$^+$ CD4$^+$ cells after parturition, that was detected by 3 panels as populations 7.1, 7.2, and 34. Among the 32 populations some change during gestation and rebound after parturition, so that no difference is observed between early gestation and post-

partum, representing transient gestation-associated perturbations. Other populations do differ between the early gestation and post-partum timepoints, indicating persistent perturbations as a result of either gestation or parturition (Fig. 2A).

Transient gestation-associated changes in T cell polarization

A transient bias in T cell polarization is observed during gestation that resolves rapidly after parturition. Notably, Th1 and Th17 cell frequencies decrease during the period of gestation, between visits 1 and 3 (Fig. 2A). This occurs not only for $CD4^+$ T helper cells generally, but also for $CD4^+$ $CXCR5^+$ T follicular helper populations (Tfh). The Tfh compartment shows significantly decreased frequencies of cells with type 1/17 phenotype, defined by CXCR3 and CCR6 expression, and significantly increased frequencies of type 2 cells lacking both of these markers (Fig. 2B,C). The shift in polarization is also observed for $CD8^+$ T cells, with significantly decreased frequencies of $CXCR3^+CCR6^-$ type 1 cytotoxic T cells during gestation (Fig. 2A). CXCR3 expression by CD8+ T cells can enable recruitment to sites of inflammation, for example by virus-specific cells in acute infection (16). Almost all of the T cell polarization phenotypes that change significantly during gestation between visits 1 and 3, change significantly in the opposite direction between visits 3 and 4 which span parturition, so that significant differences do not remain between the early gestation and post-partum timepoints (Fig. 2A).

Persisting perturbation post-parturition

In contrast to the rapidly resolving polarization changes, other differences in T and B cell populations were observed between the extreme timepoints of our study, the early gestation and post-partum visits 1 and 4 (Fig. 2A). Strikingly, the longitudinal profile of these persistent perturbations differed between T and B cell populations. T cell subsets showed few changes between early and late gestation, other than in polarization, but after parturition numerous populations differed compared to either timepoint during gestation. During this period, $CD8^+$ T

cells skew from naïve CCR7$^+$ CD45RA$^+$ cells, to terminal effector CCR7$^-$ CD45RA$^+$ or effector memory CCR7$^-$ CD45RA$^-$ cells (Fig. 2A). Changes in activation differ between CD4$^+$ and CD8$^+$ T cells, with frequency of HLA-DR$^+$ activated CD4$^+$ cells increasing, and CD38$^+$ activated CD8$^+$ cells decreasing (Fig. 2A). In contrast to these T cell changes, B cell populations that differ between visits 1 and 4 do show significant change before the end of gestation. Frequencies of transitional and activated naïve B cells decreased between visits 1 and 3, before rebounding more strongly during parturition, resulting in frequencies significantly higher post-partum compared to early gestation (Fig. 2A,D). Together these observations delineate impacts of pregnancy on the immune system that persist from early gestation to beyond 10 weeks after parturition.

Serum protein characterisation

Peripheral blood serum proteins were measured using the SomaLogic platform to quantify 1305 different proteins, in samples matching those used for flow cytometry. Longitudinal paired tests comparing between early and late gestation (visits 1 and 3) identified 434 proteins that differed significantly ($q<0.05$). We observed no correlations between the cell populations and serum proteins that were identified to change during this period (Suppl Fig. 1). This indicates that the circulating proteins and cell phenotypes behave differently and represent independent datasets. We also detected no evidence that either the cell populations or serum proteins identified to change during gestation, differed significantly between individuals in correlation with parity, maternal age, prior miscarriage, duration of gestation, body mass index or blood pressure (Suppl Tab. 3). This is likely due to comparison of a small number of pregnancies with little variation in parameters such as duration of gestation.

Two other studies have recently been reported, using a similar approach for serum proteomic measurement throughout normal pregnancy (17, 18). Romero *et al.* found that 10% of the proteins analysed changed in abundance as a function of GA, and Aghaeepour *et al*. used elastic

net (EN) modelling to identify 74 proteins that could predict GA, as well as a reduced subset of 8 which was similarly effective (17, 18). We first set out to validate these findings, by testing if the reported protein sets could be used to predict GA, using the measurements for these proteins in our cohort. Excluding 4 proteins which failed quality control parameters in our assay, we used the 70 and 8 protein sets defined by Aghaeepour *et al*., to generate EN models predicting GA. The models were trained on 80% of randomly selected samples and tested on the remaining 20%. For both protein sets, highly significant correlations were seen between observed and predicted GA ($r>0.9$, $p=0.0004$) (Fig. 3A,B, Suppl Fig. 2). Thus our results validate the serum proteins reported to predict GA in an independent dataset.

Identification of more clinically tractable predictors of gestational age

Given the predictive analyses possible using serum protein measurements, and the changes in cell population frequencies observed throughout gestation, we sought to predict GA using more accessible features such as a minimal protein sets or routinely available clinical data. Excluding the 70 proteins defined by Aghaeepour *et al*., we repeated generation of an EN model, and find that prediction of GA can still be observed ($r>0.9$, $p=0.0004$) (Fig. 3C). 7 proteins selected with highest average weight were all significantly different from null models: DDR1, PLAU, MRC1, ACP5, ROBO2, IGF2R, and GNS (Fig. 3D). In pairwise comparisons with the set of 8 proteins identified by Aghaeepour *et al*., the 7 proteins newly identified show some correlations but also substantial differences, indicating that these protein sets behave somewhat differently and do not directly mark each other (Fig. 3E). From this comprehensive serum analysis, we therefore identify a novel set of 7 proteins that can be quantified to predict GA.

Given the widespread changes throughout gestation that we observed in cell population frequencies by high parameter flow cytometry, we investigated if GA could be predicted from immune population frequencies determined at the much lower resolution of standard clinical

phenotyping. We applied the same EN modelling approach employed for predictive analysis with proteomic data, using all 33 subjects and the 3 timepoints during gestation. Models were now generated using routine clinical phenotyping measurements from CBC tests, and clinical flow cytometry that additionally quantifies 5 major populations of T, B and NK cells (Suppl Table 4). Using 19 parameters from both CBC tests and clinical flow cytometry, models predicting GA were identified with significant correlation between predicted and observed GA ($r=0.45$, $p=0.0004$) (Fig. 3F). Features that associated with GA were increase in absolute counts of monocytes, and decrease in B and NK cell absolute counts, as well as decrease in hemoglobin level (Fig. 3G). We did detect significant changes in total monocyte and NK populations by high parameter flow cytometry, where specifically $CD14^+$ CD16dim monocytes, and activated NK cells, altered most as a fraction of their parent populations (Fig. 2A). Changes in peripheral NK cell frequencies throughout gestation are not well established in previous studies, although CD56 bright NK cells show extraordinary enrichment to dominate the decidual leukocyte population at the time of implantation, these cells are transcriptionally distinct from circulating NK cells (19-21). An increase with GA has previously been reported for the total monocyte population (20). This population can be quantified by a CBC test without clinical flow cytometry and indeed, when using only 9 parameters measured from the CBC test alone, models predicting GA could still be identified where monocyte counts and hemoglobin levels were the dominant features ($r=0.35$, $p=0.0012$) (Suppl Fig. 3). These results support the idea that simple and cost effective routine clinical phenotyping measurements could be used to predict GA.

Discussion

We report high dimensional analyses characterising immune cell populations and the serum proteome, in maternal peripheral blood sampled longitudinally during normal pregnancy. In both the immune populations and serum proteome, we validate previously reported phenotypic changes, and also add new details and markers to the changes in maternal peripheral immunity

that have been identified. Expanding the changes defined adds to our understanding of the physiologically significant modifications of maternal immunity that occur during normal pregnancy, and may help the design of maternal immunization strategies.

During gestation transient T cell polarization from Th1/17 to Th2 was observed, not only for $CD4^+$ T helper cells generally but for specifically $CXCR5^+$ T follicular helper cells and also $CD8^+$ T cell subsets. T cell polarization has long been described to alter during pregnancy, with changes documented in Th1/2/17 subsets of the broad T helper peripheral $CD4^+$ population, although sometimes with contradictory results (10, 22, 23). Our data support a peripheral polarization bias from Th1/17 to Th2, but that the effects are transient with the post-partum timepoint no different from early gestation. Our novel observations that Tfh and even $CD8^+$ cells show this polarization could potentially modify how these polarization shifts are thought to alter responses to viral infections and autoantigens, which have often focused on Th cells (24-26). Tfh cells are important for vaccine responses and the changes in this population may be particularly relevant for optimization of maternal vaccination (27).

Other cellular phenotypic changes showed differences in comparisons between early gestation and post-partum timepoints. The dynamics of T cell changes, observed particularly in frequencies of $CD8^+$ cell subsets, and more widely in terms of activation status, was consistent with previous reports that broad $CD4^+$ or $CD8^+$ T cell populations show persistent alteration relative to timepoints from early gestation, in measurements of cell counts and in vitro cytokine production (20). Although immune activation has been implicated in the onset of parturition, the observation that more than 10 weeks after parturition $CD4^+$ T cell activation is still increased compared to our early gestation timepoint, could potentially impact responses in diverse scenarios such as infection, autoimmunity, and pathologies associated with chronic inflammation (28-30). It is possible the changes in cell populations could impact transfer of passive immunity to breast fed neonates, as

particularly $CD8^+$ T cells have been observed to survive in intestinal Peyer's patches of nursed infants (31). B cell populations showed a different profile of changes, with population frequencies and activation states decreasing during gestation before rebounding more strongly after parturition, to result in levels higher post-partum compared to early gestation. Our observations are consistent with previous reports that transitional B cells increase within days after parturition (32). Changes throughout the B cell compartment could derive from endocrine factors, as hormones such as human chorionic gonadotropin have been implicated in modifying B cell differentiation and function (33). These changes in B cells could contribute to mechanistic understanding of some of the specific alterations in immunity that are observed during pregnancy, such as reduced response to influenza vaccination (4). In addition to changes in population frequencies it will be important to understand alterations in cellular functional responsiveness during pregnancy, as intracellular signaling markers at endogenous levels and in response to stimulation have been found to dominate modelling of GA (14).

We applied an EN modelling approach to validate sets of 70 and 8 serum proteins sets previously reported to predict GA, and to identify a novel set of 7 serum proteins that can be used to predict GA. The new serum protein marker of GA with highest average weight was Epithelial Discoidin Domain-Containing Receptor 1 (DDR1), which is known to be involved in mammary gland development (34). DDR1 has previously been associated with birthweight in the context of disparate genome-wide approaches. Analyses of selection in primate evolution, and differential methylation between pre and full-term birth in cord blood, both implicated the region encoding DDR1 (35, 36). Others among the newly identified markers of GA have also been previously associated with pregnancy. Urokinase-Type Plasminogen Activator (PLAU) is involved in extravillous trophoblast migration during placentation, detected to be upregulated in normal pregnancy, and associated with pregnancy disorders (37-39). Cation-Independent Mannose-6-Phosphate Receptor, also known as Insulin Like Growth Factor 2 Receptor (IGF2R), was one of

the first genes identified to be imprinted in mice although in humans only a minority of individuals show biased expression from the maternal allele (40, 41). This modelling of GA is restricted to the period from 11-37 weeks for which samples were collected, it would be of significant interest to characterize earlier timepoints of gestation with prospectively collected cohorts.

In addition to the novel set of 7 serum proteins, we also show that GA can be predicted from the routinely acquired clinical data of CBC tests, and clinical flow cytometry for 5 major immune populations. The major features selected in these models were monocyte counts and hemoglobin levels. These models may be investigated in pregnancies with pathologies, to potentially shed light on the mechanisms conferring risk. Pre-term labour, for example, has been shown to be predicted by cell free RNA, white blood cell counts and corticotrophin-releasing hormone, but premature rupture of membranes remains the most accurate indicator of delivery within 48 hours (42, 43). Although much larger studies will have to be done before these observations can be adapted clinically, the ability to infer GA from standard CBC test and clinical flow cytometry measurements could be most valuable in resource poor settings. In these circumstances obstetric ultrasound is often not possible to monitor fetal growth, for detection of abnormalities that indicate increased risk of neonatal mortality (44).

Methods

Subjects and clinical phenotyping

Subjects were included in this study with a pregnancy of GA between 10-20 weeks, estimated by ultrasound or LMP, and maternal age between 18-45 years. Exclusion criteria were a hemoglobin reading of less than 8g/dL, or any medical condition adversely affecting the immune system or requiring immunomodulating medications. In total 33 individuals were studied of which 76% described ethnicity as white and 21% reported prior miscarriage. Subjects were analysed at 4 timepoints with clinical phenotyping, high parameter flow cytometry, and serum

proteomic analysis performed for all 132 samples. Clinical phenotyping was performed in the NIH clinical center and comprised complete blood count (CBC) tests and clinical flow cytometry, together measuring counts and frequencies for platelets, $CD4^+$ T cells, $CD8^+$ T cells, $CD19^+$ cells, NK cells, neutrophils and monocytes as well as hemoglobin levels (Suppl Tab. 4).

High parameter flow cytometry

Peripheral blood mononuclear cells were isolated by Ficoll separation and cryopreserved, according to Center of Human Immunology (CHI) protocols (https://chi.niaid.nih.gov/web/new/our-research/sop.html). High parameter flow cytometry was performed using the Human Immune Phenotyping Consortium (HIPC) panels as previously described (45, 46). Briefly, five parallel 10 color panels with a total of 30 unique markers, enable detection of 79 subsets of PBMCs represented as a fraction of their parent population (Suppl Tab. 1 and 2). Staining was performed using the HIPC lyophilised antibody plates, each with up to 10 samples in addition to controls, after an additional incubation with LIVE/DEAD Fixable Blue Dead Cell Stain (ThermoFisher). Acquisition was performed with a Becton-Dickinson LSRFortessa, using DIVA 8 software, acquiring 250,000 cells for each sample. Subsequent analysis to determine population frequencies used FlowJo version 9.6.2. Compensation performed with unstained cells and compensation beads was used to aid acquisition monitoring. Subsequently a final compensation matrix was calculated using FlowJo during post-acquisition analysis. Gates used to define all 79 populations are shown for a representative sample (Suppl Fig. 4-8). For one sample, subject 008 at visit 4, high parameter flow cytometry data was not obtained.

Serum proteomic analysis

Peripheral blood serum was isolated using SST tubes and cryopreserved, according to CHI protocols (https://chi.niaid.nih.gov/web/new/our-research/sop.html). Serum proteomic analysis

used the SOMAscan 1.3k Assay (SomaLogic). This is an aptamer-based assay able to detect 1305 protein analytes, optimized for analysis of human serum (47, 48). Briefly, aptamers are short single-stranded DNA sequences modified to confer specific binding to target proteins, and can be highly multiplexed for discovery of biomarker signatures. The proteins quantified include cytokines, hormones, growth factors, receptors, kinases, proteases, protease inhibitors, and structural proteins. A complete list of analytes measured can be found at http://somalogic.com/wp-content/uploads/2017/06/SSM-045-Rev-2-SOMAscan-Assay-1.3k-Content.pdf). The assay was performed according to manufacturer specifications, with data then inspected using a web tool and subjected to quality control procedures as previously described, after which results from 1194 somamers were used for analyses (49, 50).

Statistical modelling and analysis

Statistical analysis was performed using R/Bioconductor. Longitudinal changes in PBMC populations or serum proteins were evaluated by Wilcoxon signed-rank paired test, and p-values were corrected for multiple comparison with Benjamini-Hochberg method. Modelling of GA using serum proteins or cell populations was performed by elastic net (EN) method using the eNetXplorer package varying parameter alpha between 0 (ridge regression) and 1 (lasso regression) with step 0.1 (51). For each alpha the regularization parameter lambda was selected by a cross-validation procedure optimizing correlation between observed and predicted GA. For this cross-validation the modelling was run 100 times, each time using a random 80% of samples as training set and remaining 20% as test set. The best parameter alpha was chosen for models with highest average correlation between observed and predicted GA. To estimate significance of model coefficients, eNetXplorer computes null models randomly permuting sample labels 25 times, each time with 100 random samples of training and test sets as for the model.

Data and Code Availability

The data used in this study comprising CBC tests, clinical flow cytometry, high parameter flow cytometry, and serum proteomic analysis by SomaLogic; as well as the R code and documentation to reproduce the results of our analyses, are available at https://github.com/kotliary/pregnancy.

Study approval

For this study volunteers were enrolled in 10-I-0205, a NIH protocol approved and monitored by the NIAID/NIH Institutional Review Board (Bethesda, MD). All subjects provided informed consent prior to their participation in the study.

Author contributions

LB, SMO, HZ and RS recruited subjects and collected samples. JC, AB and AB performed experimental assays. RA, YK, FC and KLH analysed the data. RA, YK, YB and JST wrote the manuscript. SMH, YB, JST and CSZ conceived and oversaw the study.

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Tables

	Minimum	Mean	Maximum
Age (years)	25	33	42
Parity	1	2	5
Gestation duration (weeks)	36	39	42
Body mass index at visit 1 (kg/m^2)	19	26	43
Systolic blood pressure at visit 1 (mmHg)	91	114	139
Diasystolic blood pressure at visit 1 (mmHg)	44	64	83

Table 1: Epidemiological and physiological characteristics of the 33 individuals studied.

Figure legends

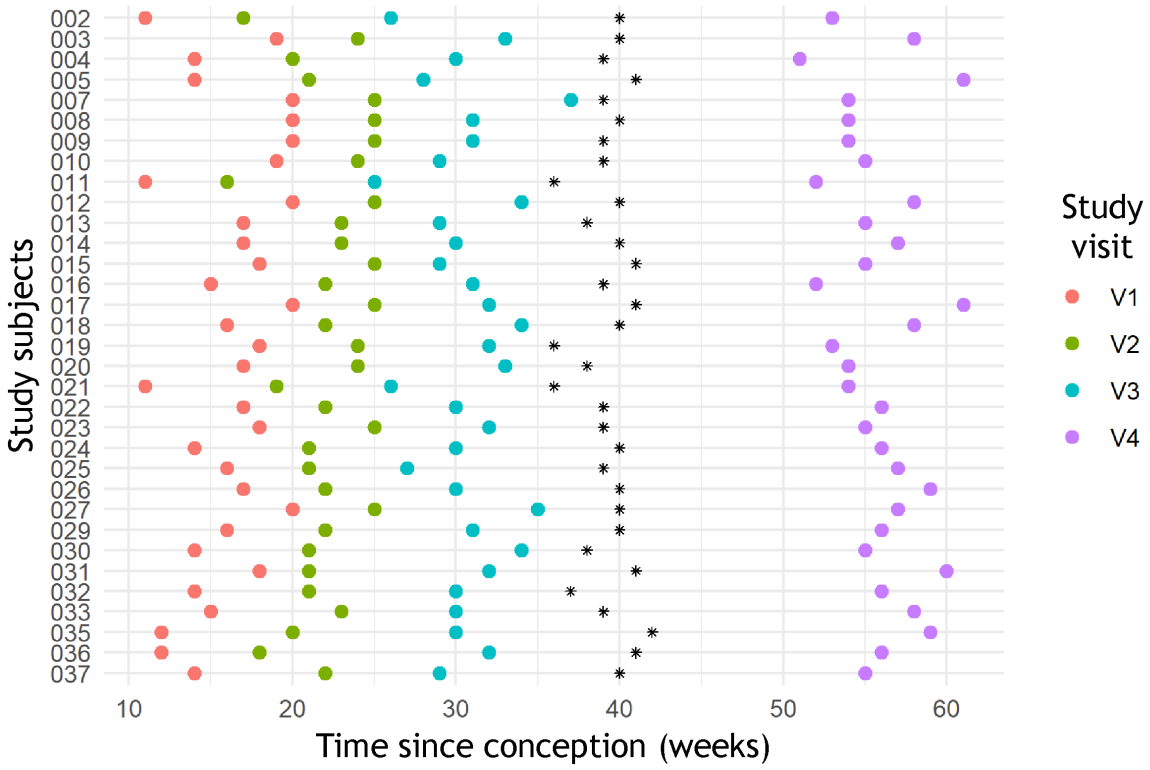
Fig. 1: Distribution of timepoints sampled in the study population. For all 33 individuals the timepoints sampled are shown for 3 visits during gestation, and one visit after parturition, with parturition marked as a black asterisk.

Fig. 2: Changes in peripheral blood immune populations throughout pregnancy. Using all 33 subjects in our study longitudinal comparisons between visits 1 and 3 (early and late gestation), visits 3 and 4 (late gestation and post-partum), or visits 1 and 4 (early gestation and post-partum) identify 32 subsets of immune cell populations with fold change greater than 1.2 or less than 0.8 and significant differences for at least one comparison using Wilcoxon signed-rank paired tests (* FDR<0.05, ** FDR<0.01, *** FDR<0.001) (A). For representative populations that demonstrate different patterns of change, fold changes compared to visit 1 are shown for all 4 visits studied. Type 2 $CD4^+$ T follicular helper cells increase during gestation and decline after parturition (B). Type 1/17 T follicular helper cells decrease during gestation and rebound after parturition (C). Transitional B cells decline significantly during gestation, but increase much more strongly after parturition (D).

Fig. 3: Serum proteins and cell populations predictive of GA. EN models generated from both a set of 70 proteins, and a subset of 8 proteins previously identified to predict GA, demonstrated significant correlation between observed and predicted GA when using serum proteomic data from 3 timepoints during pregnancy for the 33 women in our study. Predicted GA is shown with mean and standard deviation of 100 model iterations randomly sampling training and test sets, with red dashed line indicating a linear regression with 95% confidential interval (A,B). Excluding these 70 proteins and selecting from the remaining 1124 serum proteins measured, EN models still showed significant correlation between observed and

predicted GA (C). For the 7 proteins newly identified to predict GA, frequency of selection and average weights differ significantly from null models, and are plotted in red showing mean and standard deviation from the 100 model iterations randomly sampling training and test sets. In grey are shown null models with mean and standard deviation from random permutation of sample labels 25 times and 100 random samplings of the training and test sets (D). The relationship between new and previously identified proteins predicting GA was assessed by pairwise Pearson correlations of protein relative intensities during gestation in our dataset. 8 proteins previously identified to predict GA by Aghaeepour et al., and the 7 proteins identified in this study, are numbered 1-8 and 9-15 respectively and defined as follows: (1) Chorionic somatomammotropin hormone, (2) Prolactin, (3) Macrophage colony-stimulating factor 1 receptor, (4) Polymeric immunoglobulin receptor, (5) Proto-oncogene tyrosine-protein kinase receptor Ret, (6) Glypican-3, (7) Granulins, (8) alpha-Fetoprotein, (9) Macrophage mannose receptor 1, (10) Tartrate-resistant acid phosphatase type 5, (11) N-acetylglucosamine-6-sulfatase, (12) Cation-independent mannose-6-phosphate receptor, (13) Epithelial discoidin domain-containing receptor 1, (14) Urokinase-type plasminogen activator, (15) Roundabout homolog 2 (E). Standard clinical phenotyping measurements from CBC tests and clinical flow cytometry can also be used to predict GA. For the 33 subjects sampled at 3 timepoints during pregnancy, 19 routinely acquired clinical parameters were used to generate EN models for which predicted and observed GA correlated significantly, with black dashed line marking equal GA (F). Frequency of selection and average weights are shown for the significant model features (G).

Figure 1


Study subjects
002
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60
Time since conception (weeks)
Study visit
V1
V2
V3
V4

Figure 2

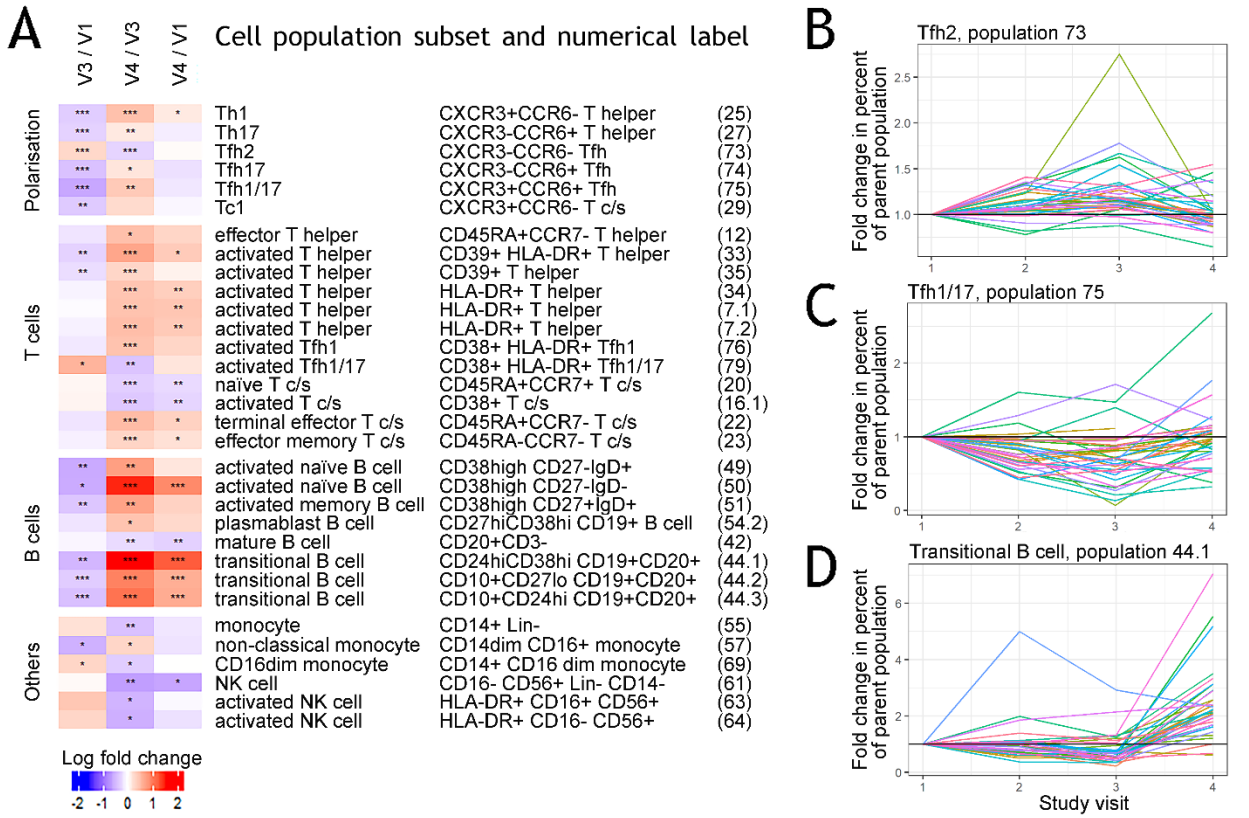
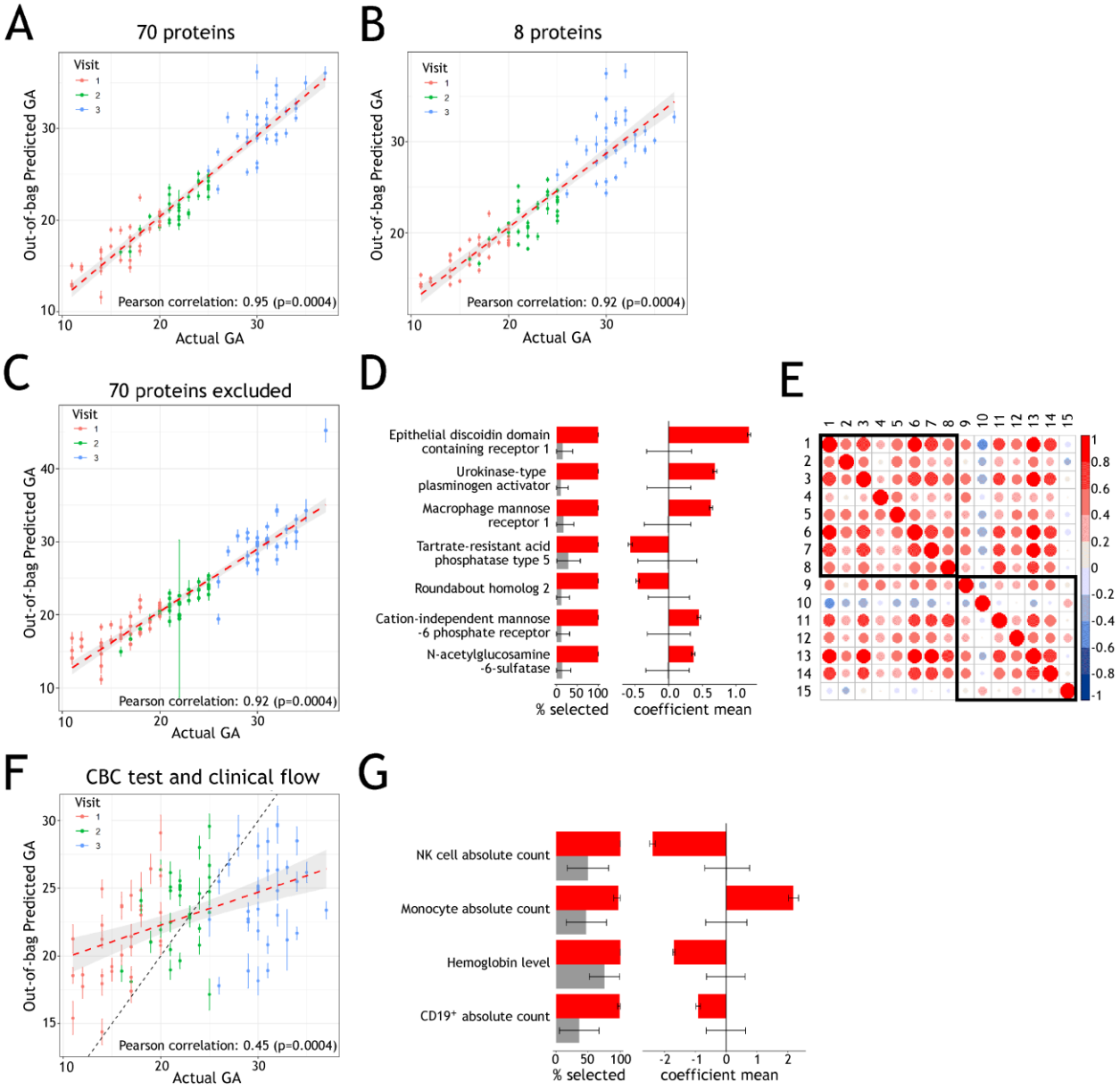

A
V3 / V1
V4 / V3
V4 / V1
Cell population subset and numerical label
Polarisation
T cells
B cells
Others
Th1 CXCR3+CCR6- T helper (25)
Th17 CXCR3-CCR6+ T helper (27)
Tfh2 CXCR3-CCR6- Tfh (73)
Tfh17 CXCR3-CCR6+ Tfh (74)
Tfh1/17 CXCR3+CCR6+ Tfh (75)
Tc1 CXCR3+CCR6- T c/s (29)
effector T helper CD45RA+CCR7- T helper (12)
activated T helper CD39+ HLA-DR+ T helper (33)
activated T helper CD39+ T helper (35)
activated T helper HLA-DR+ T helper (34)
activated T helper HLA-DR+ T helper (7.1)
activated T helper HLA-DR+ T helper (7.2)
activated Tfh1 CD38+ HLA-DR+ Tfh1 (76)
activated Tfh1/17 CD38+ HLA-DR+ Tfh1/17 (79)
naïve T c/s CD45RA+CCR7+ T c/s (20)
activated T c/s CD38+ T c/s (16.1)
terminal effector T c/s CD45RA+CCR7- T c/s (22)
effector memory T c/s CD45RA-CCR7- T c/s (23)
activated naïve B cell CD38high CD27-IgD+ (49)
activated naïve B cell CD38high CD27-IgD- (50)
activated memory B cell CD38high CD27+IgD+ (51)
plasmablast B cell CD27hiCD38hi CD19+ B cell (54.2)
mature B cell CD20+CD3- (42)
transitional B cell CD24hiCD38hi CD19+CD20+ (44.1)
transitional B cell CD10+CD27lo CD19+CD20+ (44.2)
transitional B cell CD10+CD24hi CD19+CD20+ (44.3)
monocyte CD14+ Lin- (55)
non-classical monocyte CD14dim CD16+ monocyte (57)
CD16dim monocyte CD14+ CD16 dim monocyte (69)
NK cell CD16- CD56+ Lin- CD14- (61)
activated NK cell HLA-DR+ CD16+ CD56+ (63)
activated NK cell HLA-DR+ CD16- CD56+ (64)
Log fold change
-2 -1 0 1 2
B
Tfh2, population 73
Fold change in percent of parent population
C
Tfh1/17, population 75
Fold change in percent of parent population
D
Transitional B cell, population 44.1
Fold change in percent of parent population
Study visit

Figure 3


A
70 proteins
Visit
1
2
3
Out-of-bag Predicted GA
Actual GA
Pearson correlation: 0.95 (p=0.0004)
B
8 proteins
Pearson correlation: 0.92 (p=0.0004)
C
70 proteins excluded
Pearson correlation: 0.92 (p=0.0004)
D
Epithelial discoidin domain containing receptor 1
Urokinase-type plasminogen activator
Macrophage mannose receptor 1
Tartrate-resistant acid phosphatase type 5
Roundabout homolog 2
Cation-independent mannose -6 phosphate receptor
N-acetylglucosamine -6-sulfatase
% selected
coefficient mean
E
F
CBC test and clinical flow
Pearson correlation: 0.45 (p=0.0004)
G
NK cell absolute count
Monocyte absolute count
Hemoglobin level
CD19⁺ absolute count
% selected
coefficient mean