Vaccine: X 11 (2022) 100167

Contents lists available at ScienceDirect

Vaccine: X

journal homepage: www.elsevier.com/locate/jvacx

# Signaling differences in peripheral blood mononuclear cells of high and low vaccine responders prior to, and following, vaccination in piglets

Sean Lipsit [a,b], Antonio Facciuolo [a], Erin Scruten [a], James Wilkinson [c,1], Graham Plastow [c], Anthony Kusalik [d], Scott Napper [a,b,*]

[a] Vaccine and Infectious Disease Organization (VIDO), University of Saskatchewan, Saskatoon, SK, Canada
[b] Department of Biochemistry, Microbiology, and Immunology, University of Saskatchewan, Saskatoon, SK, Canada
[c] Department of Agricultural, Food and Nutritional Science, University of Alberta, Edmonton, AB, Canada
[d] Department of Computer Science, University of Saskatchewan, Saskatoon, SK, Canada

ARTICLE INFO

*Article history:*
Received 12 October 2021
Received in revised form 29 March 2022
Accepted 25 April 2022
Available online 9 May 2022

*Keywords:*
Vaccine Response
Kinome
Biomarker
Phosphorylation

ABSTRACT

Individual variability in responses to vaccination can result in vaccinated subjects failing to develop a protective immune response. Vaccine non-responders can remain susceptible to infection and may compromise efforts to achieve herd immunity. Biomarkers of vaccine unresponsiveness could aid vaccine research and development as well as strategically improve vaccine administration programs. We previously vaccinated piglets (n = 117) against a commercial Mycoplasma hyopneumoniae vaccine (RespiSure-One) and observed in low vaccine responder piglets, as defined by serum IgG antibody titers, differential phosphorylation of peptides involved in pro-inflammatory cytokine signaling within peripheral blood mononuclear cells (PBMCs) prior to vaccination, elevated plasma interferon-gamma concentrations, and lower birth weight compared to high vaccine responder piglets. In the current study, we use kinome analysis to investigate signaling events within PBMCs collected from the same high and low vaccine responders at 2 and 6 days post-vaccination. Furthermore, we evaluate the use of inflammatory plasma cytokines, birthweight, and signaling events as biomarkers of vaccine unresponsiveness in a validation cohort of high and low vaccine responders. Differential phosphorylation events (FDR < 0.05) within PBMCs are established between high and low responders at the time of vaccination and at six days post-vaccination. A subset of these phosphorylation events were determined to be consistently differentially phosphorylated ($p < 0.05$) in the validation cohort of high and low vaccine responders. In contrast, there were no differences in birth weight ($p > 0.5$) and plasma IFNγ concentrations at the time of vaccination ($p > 0.6$) between high and low responders within the validation cohort. The results in this study suggest, at least within this study population, phosphorylation biomarkers are more robust predictors of vaccine responsiveness than other physiological markers.


## 1. Introduction

Despite the demonstrated utility of vaccines for controlling infectious diseases in humans and animal populations, vaccination programs are sometimes challenged by inter-individual variation in vaccine-induced immune responses. This is particularly true for large populations which are heterogeneous with respect to age, genetics, and health status. Individuals who fail to develop a protective immune response against the vaccine, or "vaccine non-responders", can remain at risk for infection and may compromise the protection afforded to the population through herd immunity [19,41,50]. Variability in vaccine-induced immune responses can reflect characteristics of either the vaccine or the individual; not all vaccines are equally effective in inducing consistent immune responses and not all individuals are equally effective in eliciting a response to a given vaccine. From the perspective of the vaccine, inconsistent responses can reflect issues relating to antigen optimization [52], vaccine formulation [8,22], and vaccine administration [61]. Similarly, individual-level factors including age [11,26], body weight [43,49], health status [25], genetic polymorphisms [42,44,54], and microbiome composition [17,21] can impact individual vaccine-induced immune responses. Efforts to understand the host factors that mitigate vaccine responses have focused on the distinct, but related, activities of understanding

* Corresponding author.
*E-mail address:* scott.napper@usask.ca (S. Napper).
[1] Current Address: Illumina Centre, Great Abington, Cambridge, UK.

https://doi.org/10.1016/j.jvacx.2022.100167
2590-1362/

molecular mechanisms of vaccine responsiveness and identifying biomarkers that anticipate the extent of these outcomes [45].

Understanding the biochemical basis of vaccine responsiveness and identifying vaccine response biomarkers both rely on interpreting the complex molecular mechanisms underlying effective immunological responses to vaccination. Thus, several -omic approaches have been applied to define host responses to vaccination, but the major emphasis has been on transcriptional analysis. This includes defining gene expression events in response to vaccines against yellow fever virus [14], influenza virus [20,36,37], hepatitis B virus [10], shingles virus [27] tetanus toxoid [1], foot-and-mouth-disease virus [22], and *Mycoplasma hyopneumoniae* (*M. hyopneumoniae*) [5,29]. Defining patterns of gene expression within peripheral blood leukocytes that correlate with various quantifiable vaccine outcomes (eg. functional antibody responses, cell-mediated immune responses, or adverse effects) can be utilized for predicting these vaccine outcomes and furthers our understanding of their molecular mechanisms [15]. Molecular events following vaccination, including vaccine-induced gene expression events, have been utilized as input data for a variety of machine learning and statistical models to predict vaccine responsiveness [14,36,55]. Additionally, characterizing the baseline immune environment that modulates vaccine responses are equally valuable for discerning vaccine outcomes, yet relatively few studies have taken this approach [10,13,48,58]. Therefore, approaches capable of depicting the state of the immune environment at the time of vaccination, as well as describe the immune response to vaccination may provide a more complete perspective on the molecular events driving vaccine responsiveness.

Kinase-mediated phosphorylation of proteins is a central mechanism of regulation of cellular responses. With that, there is increasing priority to investigate phenotypes in terms of patterns of global cellular kinase activity (kinome analysis). One effective approach for kinome analysis is to utilize peptide arrays is which short (15-mer) peptides are used as surrogate substrates for cellular kinases [2]. These peptides represent specific biological phosphorylation events, in which the phosphoacceptor site is situated in the central position while maintaining the surrounding amino acid residues as present in the corresponding protein. Upon exposure of the array to a cellular lysate, the degree of phosphorylation of each peptide reflects the activity of the associated kinase. Comparing phosphorylation profiles of arrays corresponding to different biological conditions enables quantification of the relative activities of specific kinases as well as anticipation of the extent of phosphorylation of the proteins represented by the peptides [2]. Peptide arrays have proven a cost-effective, robust tool for kinome profiling. Coupled with the emergence of software that predicts the phosphoproetomes of species of interest, there is the opportunity to rapidly generate arrays that are customized with respect to species and biological process. This is particularly valuable for investigations where there is limited available of species-specific reagents [9].

Characterization of global patterns of phosphorylation-mediated signal transduction activity (kinome analysis) have proven effective for defining immunological and metabolic responses in the context of host-pathogen interactions [3,23,60], cancer [51], and stress [7,38]. In an investigation of responses of cattle to restraint stress, kinome analysis of peripheral blood mononuclear cells (PBMCs) implicated signaling events associated with carbohydrate metabolism that supported the use of plasma glucose levels as a simple, economical biomarker of stress in cattle [7]. Robertson et al. [46] established a panel of phosphorylation events implicated in innate immune signaling that was correlated with the susceptibility of honeybees to Varroa mite infestation. These phosphorylation biomarkers discriminated the susceptibility of honeybee colonies to Varroa mite infestation prior to exposure to the pathogen, demonstrating proof-of-concept that phosphorylation biomarkers have value for phenotype prediction [46,47]. Previously, our group conducted transcriptional and kinome analysis on PBMCs collected from piglets prior to *M. hyopneumoniae* vaccination to identify differences between high and low vaccine responders [28,35]. While the transcriptional analysis did not detect pre-vaccination differences in gene expression, kinome analysis revealed differential phosphorylation events in PBMCs prior to vaccination that were functionally enriched in pro-inflammatory cytokine signaling [28].

This study utilizes the previously described population of piglets vaccinated against *M. hyopneumoniae* using a commercial vaccine (RespiSure-One) to characterize signaling events within PBMCs at 2 and 6 days following vaccination. Additionally, we further evaluate the utility of birthweight, pro-inflammatory plasma cytokine levels prior to vaccination, and these signaling events as biomarkers for vaccine responsiveness. Using vaccine-induced serum immunoglobulin G (IgG) responses as the metric for vaccine responsiveness, we classify high (HR) and low (LR) vaccine responders into discovery and validation cohorts and conduct kinome analysis on PBMCs collected prior to vaccination and 2- and 6-days post-vaccination. While *M. hyopneumoniae* antibody responses are not considered to be completely protective of *M. hyopneumoniae* infection in swine [8,52], the commercial vaccine provided a valuable tool for inducing a range of antibody responses useful for examining variable vaccine responsiveness. Multiple differential phosphorylation events are identified between the HR and LR within the discovery cohort both prior to vaccination and at six days post-vaccination. Many of these differential phosphorylation events were consistent between HR and LR within the validation cohort at their respective time points. Conversely, birth weight and plasma cytokine levels failed to differentiate HR and LR within the validation cohort, suggesting that at least within this study population, signal transduction events in blood leukocytes prior to, and early after vaccination are more sensitive predictors of vaccine responsiveness than other physiological markers.

## 2. Methods

### 2.1. Animal Care and vaccination

The experimental protocol (AUP00001125) was approved by the University of Alberta Animal Care and Use Committee–Livestock in accordance with the Canadian Council on Animal Care guidelines. The animals used for this study have been described previously [28]. Briefly, piglets (n = 117) were vaccinated intramuscularly with one dose (1 mL) of RespiSure-One (Zoetis, U.S.A) at 28 days of age (Day 0) and received a booster vaccination at 52 days of age (Day 24). The trial was terminated when piglets were 63 days of age (Day 35). Bodyweight was recorded at 0-(Birth), 24- (Weaning), and 63-days of age. A nasal swab from each piglet was tested prior to vaccination on Day 0 to confirm the piglets were negative for *M. hyopneumoniae*. Maternal serological status was not measured for sows in this study, but animals within the facility had been reported as *M. hyopneumoniae*-free at the time of this study and for several years prior to this study. Sows were not vaccinated with RespiSure-One to reduce the possibility that vaccine-induced antibodies measured in piglets were of maternal origin.

### 2.2. Serum and plasma Collection, and PBMC isolation

Whole blood was collected from the jugular vein prior to primary vaccination at 28 days of age (Day 0) and following vaccination at 30 (Day 2) and 34 (Day 6) days of age in 0.4% EDTA (Sigma-

Aldrich) in $Ca^{2+}$ and $Mg^{2+}$-free phosphate-buffered saline (PBS). The protocol for PBMC isolation, serum, and plasma collection has been described previously [28].

### 2.3. Mycoplasma hyopneumoniae-specific IgG ELISA and Stratification of high and low responders

Methods for determining *M. hyopneumoniae*-specific IgG titers in serum have been described previously [28,35]. All piglets within the study population were included in the *M. hyopneumoniae*-specific IgG titer population distribution (Fig. 1A). Piglets were stratified into high and low responders using *M. hyopneumoniae*-specific IgG titers quantified at 63 days of age (Day 63). However, several piglets were excluded from high and low responder cohorts based on the limited availability of archived PBMC samples. Thus, twelve piglets for which there was sufficient sample to perform kinome profiling on all three days were selected to represent the high (n = 6) and low (n = 6) vaccine responders within the discovery cohort. Eight additional piglets with sufficient sample were selected to represent the high (n = 4) and low (n = 4) vaccine responders within the validation cohort. One piglet classified as a high responder in the validation cohort ("388B") on Day 0 did not have PBMCs collected on Day 6 and was substituted with another high responder ("893R") for the Day 6 validation analyses only.

### 2.4. Porcine-Specific magnetic multiplex cytokine analysis

The protocol for the porcine-specific magnetic multiplex cytokine assay has been described previously [28]. The lower limit of quantification (LLOQ) values for all cytokines are given: interferon-gamma (IFNγ) (14.4 pg/mL), interleukin 1-beta (IL-1β) (37.5 pg/mL), and tumor necrosis factor-alpha (TNFα) (39.8 pg/mL). Samples below the LLOQ were manually recorded as ½ the LLOQ value. IFNγ and IL-1β were measured simultaneously while TNFα was measured separately.

### 2.5. Kinome analysis

The kinome array experiments were performed as previously described with modifications [28] (Supplemental Fig. 1). Kinome array experiments for each time-point (Day 0, Day 2, and Day 6) were conducted on independent days. No difference ($p > 0.9$) in inter-array variation between experiments conducted on different days was observed (Supplemental Fig. 2). Peptide-spot phosphorylation intensities from piglets in the discovery cohort on each time-point were transformed separately from piglets in the validation cohort using a variance-stabilizing normalization (VSN) method through the online software, PIIKA (https://saphire.usask.ca/saphire/piika/) [56]. Peptide-spot phosphorylation intensities from piglets in the validation cohort at all time-points were subsequently transformed using the VSN method with the inclusion of piglets within the discovery cohort datasets to allow for comparable scales. Technical replicates were averaged together, and fold-change (FC) for each peptide phosphorylation intensities was calculated using $Log_2$ values. FC = 2^d, where d = (average intensity of group y – average intensity of group x). The negative reciprocal of FC was calculated when d < 1 for interpretation purposes.

### 2.6. Data and statistical analysis

All data analysis and data visualizations were performed using GraphPad Prism version 9.0 (GraphPad Software, San Diego, California USA, https://www.graphpad.com). Principal component analysis (PCA) was conducted and visualized using ClustVis version 1.0 with the parameters: transformation = "no transformation", row scaling = "unit variance scaling", PCA method = "SVD with imputation" [32]. $Log_2$ *M. hyopneumoniae*-specific IgG titers and body weight for discovery HR and LR were determined to be normally distributed (Kolmogorov-Smirnov test, $p > 0.1$). $Log_2$ *M. hyopneumoniae*-specific IgG titers, plasma cytokine levels, and bodyweights for the validation HR and LR could not be determined to be normally distributed due to the small sample size. A Mann-Whitney *U* test was used to determine differences in median $Log_2$ *M. hyopneumoniae*-specific IgG titers, plasma cytokine concentrations, and body weight between HR and LR cohorts. To determine differential phosphorylation events, a repeated-measures two-way ANOVA with Geisser-Greenhouse correction was conducted using the average VSN-transformed intensity for each phosphorylation event with the factors "Day" and "Response". Sidak's multiple comparisons were conducted between HR and LR for each Day. A Benjamini-Hochberg False Discovery Rate (FDR)-Correction (5%) was applied to p-values on each Day. Phosphorylation events were considered differentially phosphorylated between high and low responders under two criteria: there was an effect ($p < 0.05$) of either the "Response" factor or the "Response × Day" interaction, and there was a difference (FDR < 0.05) between HR and LR after FDR-correction. Phosphorylation events were considered differentially phosphorylated between Day 0, Day 2, or Day 6 under two criteria: there was an effect ($p < 0.05$) of either the "Day" factor or the "Responder × Day" interaction, and there was a difference (FDR < 0.05) between Day 0 and Day 2, Day 0 and Day 6, or Day 2 and Day 6 after FDR-correction. Mann-Whitney U-tests were used to determine differences in median phosphorylation intensity between HR and LR in the validation cohort. P-values and FDR-values were considered statistically significant at $p < 0.05$ and FDR < 0.05, respectively.

## 3. Results

### 3.1. Stratification of discovery and validation cohorts using Vaccine-induced Mycoplasma hyopneumoniae-specific IgG titers

A population of piglets (n = 117) were vaccinated with a commercial *M. hyopneumoniae* vaccine (RespiSure-One) at 28-days of age (Day 0) and given a booster at 52-days of age (Day 24). Serum *M. hyopneumoniae*-specific IgG titers were quantified at 63-days of age (Day 35), eleven days following booster vaccination, using an IDEXX *M. hyo* Ab ELISA (Fig. 1A). Within this population, serum IgG titers of high (HR) responders from the 90th percentile (n = 6; range, 12.13–13.67; median, 12.81) and low (LR) responders from the 10th percentile (n = 6; range, 5.85–7.65; median, 7.19) were used to establish the "discovery cohort". Due to limited sample availability piglet "HR5" was substituted with a different piglet within the 90th percentile of serum IgG titers ("HR7") for subsequent analyses [28]. Between the HR and LR in the discovery cohort there was a 48-fold difference ($p < 0.01$) in median *M. hyopneumoniae*-specific serum IgG titers (Fig. 1B). A second subpopulation of HR from the 80th percentile of serum IgG titers (n = 4; range, 11.95–12.21; median, 11.97) and LR from the 20th percentile of serum IgG titers (n = 4; range, 7.84–8.04; median, 7.96) were used to establish the "validation cohort". There was a 16-fold difference ($p < 0.01$) in median *M. hyopneumoniae*-specific IgG titers between the validation HR and LR (Fig. 1B). The HR within the validation cohort had a lower rank-sum difference (Mann-Whitney *U* test, $p < 0.05$) in log2 *M. hyopneumoniae*-specific IgG titers compared to HR within the discovery cohort. Conversely, LR within the validation cohort had a higher rank-sum difference (Mann-Whitney *U* test, $p < 0.01$) in log2 *M. hyopneumoniae*-specific IgG titers than the discovery LR. Thus, high

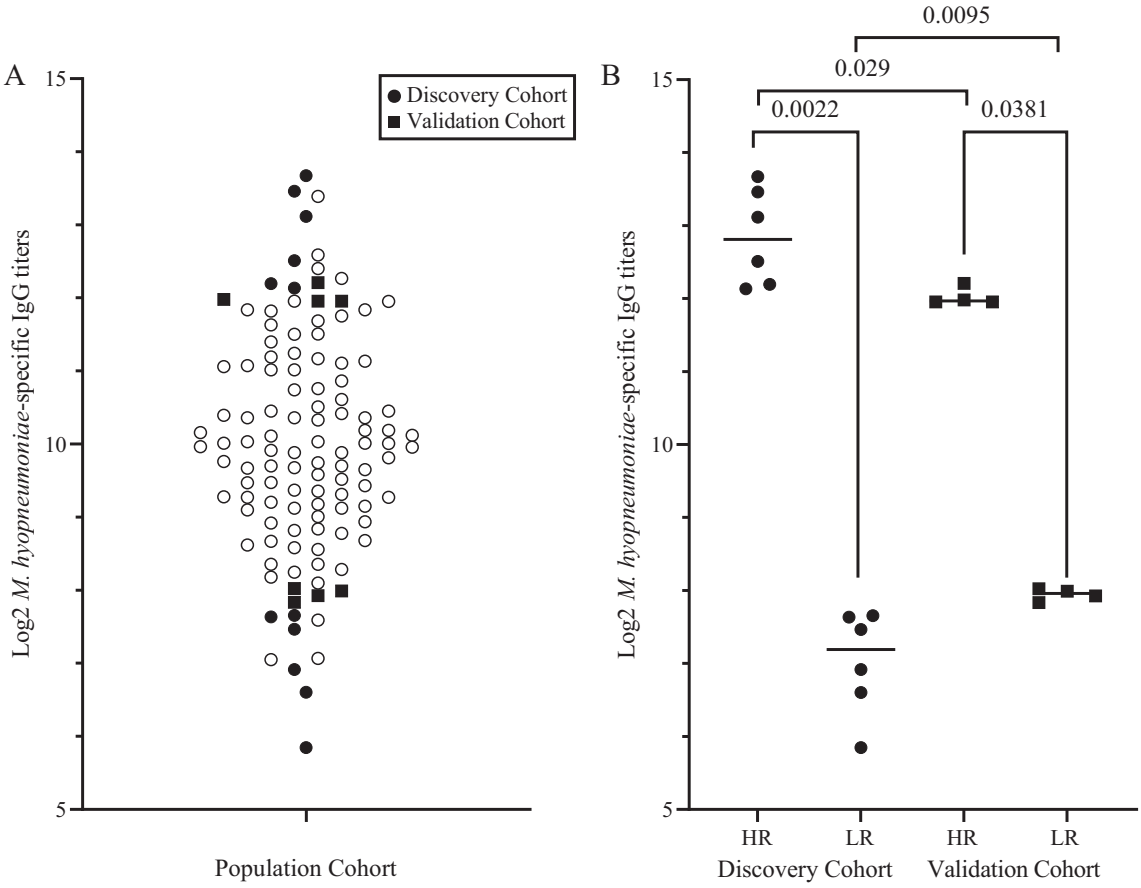


**Fig. 1. Stratification of high and low responder piglets using *Mycoplasma hyopneumoniae*-specific IgG responses 11 days following booster vaccination**. **A.** *M. hyopneumoniae*-specific IgG titers ($log_2$) in the study population (n = 117). High and low responders from two sampling subsets, the discovery (n = 12; filled circles) and validation (n = 8; filled squares) cohorts are denoted. **B.** Median *M. hyopneumoniae*-specific IgG titers ($log_2$) of high (HR) and low (LR) responders within the discovery cohort (n = 6/group; filled circles) and the validation cohort (n = 4/group; filled squares). P-values were determined using the Mann-Whitney U tests.

and low responders within the validation cohort represent a less extreme phenotype of vaccine-induced IgG responses compared to the discovery cohort.

### 3.2. Comparison of plasma cytokine concentrations and birthweight between low and high responders

Previously, we observed that LR piglets within the discovery cohort had higher plasma concentrations of IFNγ ($p < 0.05$), IL-1β ($p = 0.06$), and TNFα ($p = 0.12$) on Day 0 when compared to HR of the discovery cohort [28] (Fig. 2A). In the current study, there was no statistical difference in plasma concentrations of IFNγ ($p > 0.60$), IL-1β ($p < 0.99$), or TNFα ($p < 0.99$) on Day 0 between the LR and HR within the validation cohort (Fig. 2B). When comparing bodyweights, HR within the discovery cohort had a higher birth weight ($p < 0.01$) and weaning weight ($p < 0.05$) compared to LR of the discovery cohort (Fig. 2C) [28]. In contrast, there was no difference in either birth weight ($p > 0.5$) or weaning weight ($p > 0.5$) between the HR and LR of the validation cohort (Fig. 2D). These data suggest that plasma cytokine concentrations and physiological differences in bodyweight do not correlate with vaccine responsiveness as the magnitude of vaccine responses becomes less extreme.

### 3.3. Kinome analysis of discovery cohort on Day 0, 2, and 6

To determine the phosphorylation events within blood leukocytes associated with vaccine-induced antibody responses, kinome analysis was performed on PBMCs collected from HR and LR immediately prior to vaccination (Day 0) and 2- and 6- days post-vaccination (Days 2 and 6). Kinome profiles were generated using the 282 phosphorylation events represented on the peptide array and principal component analysis (PCA) was conducted on HR and LR for each time-point. Prior to vaccination on Day 0, there is a high intra-group similarity between LR piglets, whereas the HR had a greater amount of variability when considering all 282 phosphorylation events represented of a kinome profile (Fig. 3A). Kinome profiles on Day 2 do not perfectly cluster based on vaccine responsiveness phenotypes and have overlapping 95% confidence intervals between LR and HR kinome profiles (Fig. 3B). Finally, PCA of Day 6 kinome profiles reveals PC1 (23.3%) was capable of separating HR and LR kinome profiles, suggesting multiple phosphorylation events can differentiate HR and LR (Fig. 3C). We then compared individual phosphorylation events between HR and LR within the discovery cohort at each time-point to determine specific biomarkers that strongly associate with vaccine responsiveness.

### 3.4. Differential phosphorylation events within discovery cohort

To identify the unique phosphorylation events between HR and LR within the discovery cohort at each time-point, a repeated-measures two-way ANOVA using the factors "Response" (High vs Low) and "Day" (Day 0 vs Day 2 vs Day 6) was conducted using the 282 phosphorylation intensities from the kinome analysis. ***Day 0:*** Ten differential phosphorylation events (FDR < 0.05) on Day 0 were detected between the HR and LR of the discovery

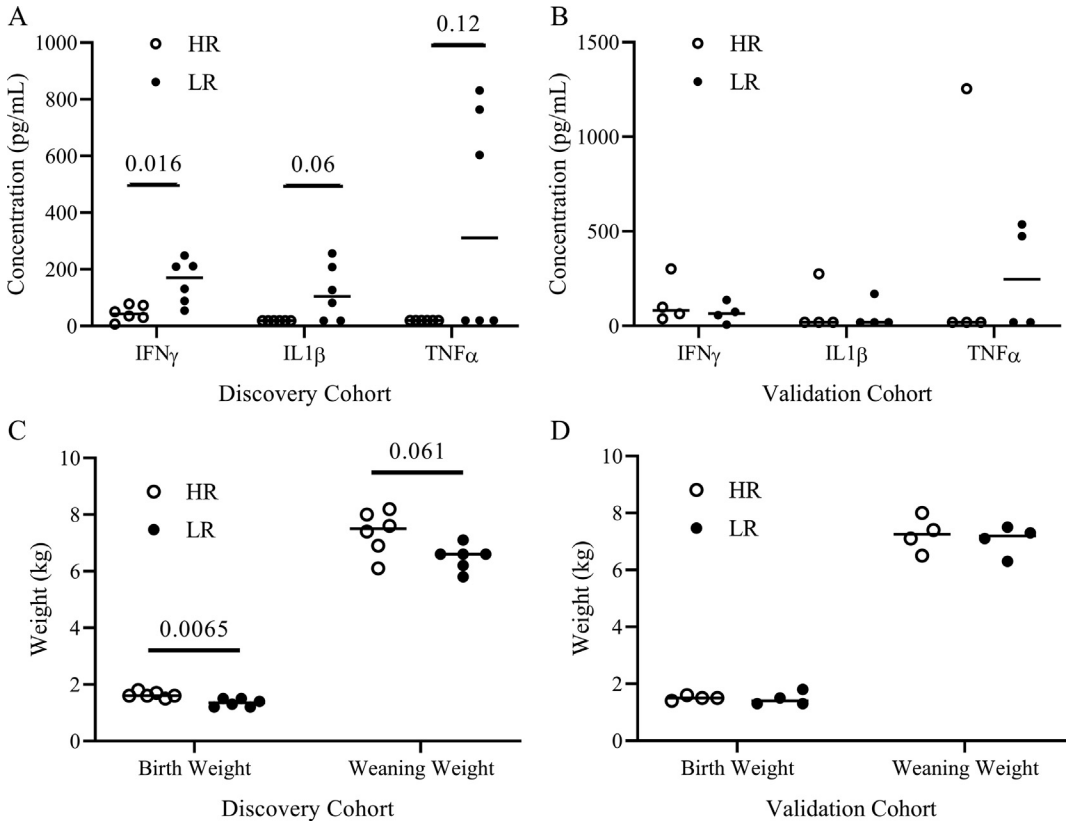


**Fig. 2. Plasma cytokine concentrations prior to vaccination and bodyweights at birth and weaning in the discovery and validation cohorts. (A)** Discovery (n = 6/group) and **(B)** validation (n = 4/group) cohorts plasma cytokine concentrations (pg/mL) of interferon-gamma (IFNγ), interleukin 1-beta (IL-1β), and tumor necrosis factor-alpha (TNFα) within high (HR; empty circles) and low (LR; filled circles) responders prior to vaccination. The horizontal line represents the group median. **(C)** Discovery (n = 6/group) and **(D)** validation (n = 4/group) cohorts bodyweight (kg) at birth and weaning of HR (empty circles) and LR (filled circles) for the. The horizontal line represents the group median. P-values were determined using the Mann-Whitney U tests.

cohort (Table 1). Among the peptides differentially phosphorylated between HR and LR on Day 0 there were peptide targets representing mediators of immune-function such as B-cell linker protein (BLNK), interleukin 6 receptor (IL6ST), tumor necrosis factor receptor-associated factor 6 (TRAF6), and cell signaling mediators such as AKT1, protein phosphatase 2 catalytic subunit alpha (PPP2CA), and calmodulin (CALM1). Eight of the 10 phosphorylation events had higher phosphorylation (FC > 1) in LR compared to HR. ***Day 2:*** There were no individual phosphorylation events detected as significantly different on Day 2 between the discovery HR and LR at an FDR of 5% (Table 1). ***Day 6:*** Eleven differential phosphorylation events (FDR < 0.05) on Day 6 were detected between the HR and LR of the discovery cohort (Table 1). Among the Day 6 differential phosphorylation events, there were peptide targets representing proteins involved in cell signaling mediation like phosphoinositide-3-kinase regulatory subunit 1 (PIK3R1) and receptor of activated C kinase 1 (RACK1), cytoskeletal proteins like stathmin 1 (STMN1) and PPP2CA, and proteins with known immunological signaling functions like TRAF6, SYK, and nuclear factor of activated T-cells 2 (NFAT2). Seven of the 11 differential phosphorylation events had higher intensity (FC > 1) in the LR compared to the HR. In comparing the biomarkers detected on Day 0 and 6, three differential phosphorylation events on identical target sites, TRAF6_Y353, STMN1_S15, and PPP2CA_T304, were temporally consistent with similar fold-changes at each time-points.

### 3.5. Phosphorylation biomarkers in the validation cohort (Day 0)

To validate the robustness of the differential phosphorylation events discovered on Day 0 and 6, we then tested the capability of the phosphorylation events to discriminate a set of high and low vaccine responders from a validation cohort using both PCA and comparative analysis. On Day 0, PCA of the 282-peptide kinome profiles from the HR and LR within the validation cohort do not perfectly separate individuals based on vaccine responsiveness phenotype (Fig. 4A). Piglets within the validation cohort were given new IDs to blind their vaccine response phenotypes and only the phosphorylation intensities of the 10 differential phosphorylation events on Day 0 (described in Table 1) were used in a subsequent PCA. Using the 10 differential phosphorylation events identified in the discovery cohort, PC1 (52%) is capable of reducing the inter-group overlap of 95% confidence intervals (Cis) between HR and LR within the validation cohort in comparison to the untargeted 282-peptide kinome profile (Fig. 4B).

We then determined if the 10 differentially phosphorylated peptides identified in the discovery cohort on Day 0 were similarly differentially phosphorylated between the HR and LR within the validation cohort. Comparative analysis revealed multiple phosphorylation events from piglets within the validation cohort had similar magnitude and direction of changes in phosphorylation as observed in the discovery cohort (Fig. 4C). Phosphorylation events such as STMN1_S15, TRAF6_Y353, CALM1_Y99, and PPP2CA_T304, were consistently differentially phosphorylated ($p < 0.05$) within the validation cohort, while the remaining phosphorylation events had no difference ($p > 0.05$) between HR and LR in the validation cohort. Altogether, multiple phosphorylation events observed in the discovery cohort on Day 0 persist between HR and LR within the validation cohort. As differences in plasma cytokine concentrations (Fig. 2B) or body weight (Fig. 2D) had

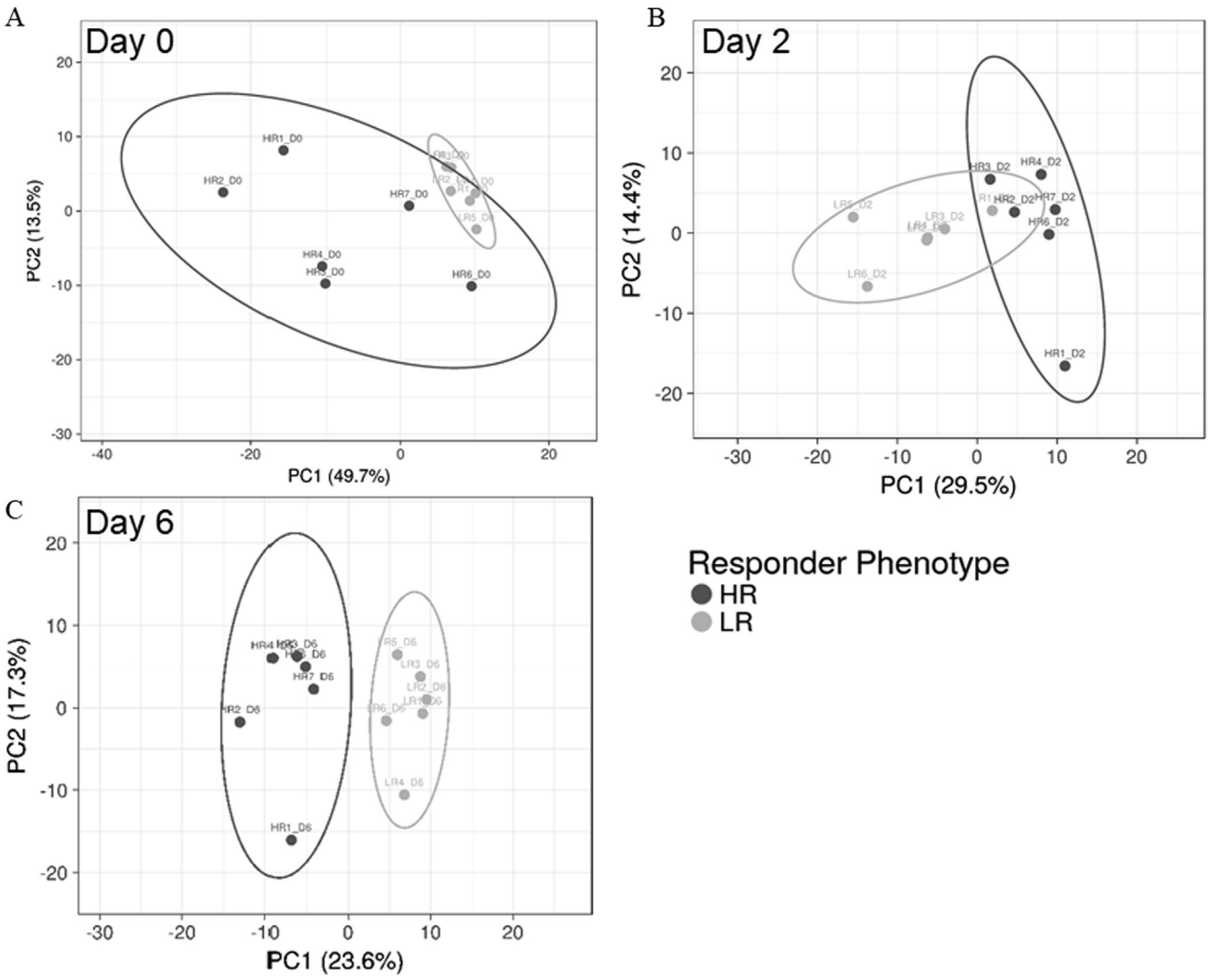


**Fig. 3. Principal component analysis of the high and low responders within the discovery cohort** Principal component (PC) analysis of high (HR; n = 6; dark grey) and low (LR; n = 6, light grey) responders using phosphorylation events of 282 peptides on **(A)** Day 0, **(B)** Day 2, and **(C)** Day 6. The two PCs with the highest variance (%) are shown. Ellipses represent 95% confidence intervals for each group.

failed to differentiate HR and LR of the validation cohort prior to vaccination, these data suggest phosphorylation patterns have greater consistency in associating with vaccine-induced antibody responses at the time of vaccination.

### 3.6. Phosphorylation biomarkers in the validation cohort (Day 6)

To test the robustness of the differential phosphorylation events between HR and LR within the discovery cohort on Day 6 for discriminating vaccine responders, we repeated the analyses conducted on the Day 0 kinome profiles using the Day 6 kinome profiles of the validation cohort. One piglet classified as HR in the validation cohort ("388B") on Day 0 did not have PBMCs collected on Day 6 and was substituted with another HR ("893R") for subsequent Day 6 validation cohort analyses. PCA of the HR and LR within the validation on Day 6 using the entire 282-peptide kinome profile revealed indistinct clustering of HR and LR, suggesting a random profile of peptide phosphorylation events are incapable of differentiating high and low vaccine responders (Fig. 5A). Reducing the consideration to the 11 phosphorylation events identified in the discovery cohort on Day 6 (described in Table 1) reveals that a combination of PC1 (45%) and PC2 (21.7%) can separate HR and LR within the validation cohort (Fig. 5B). Comparative analysis of the 11 phosphorylation events that were differentially phosphorylated between HR and LR within the discovery cohort on Day 6 were consistently different between HR and LR within the validation cohort (Fig. 5C). Specifically, the phosphorylation events STMN1_S15 and Nuclear factor of activated T-cells 2 (NFAT2)_S245 are differentially phosphorylated ($p < 0.05$) between the validation HR and LR, while Kelch-Like ECH-Associated Protein 1 (KEAP1)_Y293/5, and SYK_Y348 had a trend ($p = 0.11$) toward differential phosphorylation. All these phosphorylation events demonstrate highly similar patterns of direction and magnitude of change in the discovery and validation cohorts. There were no suspected differences ($p > 0.11$) between HR and LR within the validation cohort for the remaining biomarker phosphorylation events, indicating thresholds of limitation for discriminating less extreme vaccine responders.

### 3.7. Temporal changes in phosphorylation in the discovery cohort

To investigate the changes of phosphorylation events within PBMCs following vaccination, a repeated-measures two-way ANOVA with the factors "Response" (High vs Low) and "Day"

**Table 1**
Differential phosphorylation events within PBMCs between low and high responders on Day 0, Day 2, and Day 6 in the discovery cohort.

| | Target Name | Target Site | UniProt ID | FC[a] | FDR[b] q-value |
|---|---|---|---|---|---|
| **Day 0** | STMN1 | S15 | P16949 | −1.89 | 0.0143 |
| | TRAF6 | Y353 | Q9Y4K3 | −2.33 | 0.0143 |
| | BLNK | Y178 | Q8WV28 | 1.55 | 0.0163 |
| | CALM1 | Y99 | P0DP23 | −1.66 | 0.0163 |
| | FGFR1 | Y653 | P11362 | −1.70 | 0.0163 |
| | IL6ST | S782 | P40189 | 1.75 | 0.0163 |
| | PPP2CA | T304 | P67775 | −2.04 | 0.0163 |
| | AKT1 | T308 | P31749 | −2.10 | 0.0214 |
| | STAT4 | S722 | Q14765 | −1.88 | 0.0254 |
| | RPS6KB1 | S447 | P23443 | −1.62 | 0.0400 |
| **Day 2** | – | – | – | – | – |
| **Day 6** | STMN1 | S15 | P16949 | −1.62 | 0.0142 |
| | TRAF6 | Y353 | Q9Y4K3 | −2.16 | 0.0142 |
| | PPP2CA | T304 | P67775 | −1.96 | 0.0142 |
| | NFAT2 | S245 | O95644 | 2.41 | 0.0142 |
| | STMN1 | S37 | P16949 | −2.66 | 0.0142 |
| | PIK3R1 | Y556 | P27986 | −1.65 | 0.0142 |
| | SMAD1 | S214 | Q15797 | 1.40 | 0.0142 |
| | RAB5A | T202 | P20339 | −1.84 | 0.0142 |
| | RACK1 | Y194 | P63244 | −1.80 | 0.0253 |
| | SYK | Y348 | P43405 | 1.48 | 0.0362 |
| | KEAP1 | Y141 | Q14145 | 1.27 | 0.0362 |

[a] Fold-change (FC) is calculated as a change from low responders (x) to high responders (y).
[b] False-discovery rate (FDR) was applied to Sidak's multiple comparison tests between high and low responders on each time-point. FDR was set at 0.05.

(Day 0 vs Day 2 vs Day 6) was conducted to identify temporal differences in phosphorylation within LR and HR of the discovery cohort. These differential phosphorylation changes could not be validated in the validation cohort as not all piglets in the validation cohort had paired samples.

### 3.8. Day 0 and Day 2

LR had 10 (2 increasing; 8 decreasing) unique phosphorylation changes (FDR < 0.05) while HR had zero (Table 2). ***Day 0 to Day 6:*** LR had 1 (increasing) unique phosphorylation change (FDR < 0.05) while HR had zero (Table 2). ***Day 2 to Day 6:*** LR had 21 (5 increasing; 16 decreasing) unique phosphorylation changes (FDR < 0.05) while HR had 1 (decreasing) unique phosphorylation change (FDR < 0.05) (Table 2).

Of the phosphorylation changes from Day 0 to Day 2 and Day 2 to Day 6 within LR, there are 4 that significantly change over both time-intervals: Murine double mutant 2 (MDM2)_S166, interferon regulatory factor-3 (IRF-3)_S402, STMN1_S37, and TRAF6_Y353. All of these phosphorylation events had opposite fold-changes between Day 0 to Day 2 and Day 2 to Day 6. NFAT2_S245 is the only peptide target to had differential phosphorylation among all three time intervals within LR. Cyclin Dependent Kinase Inhibitor 1B (CDKN1B)_Y74 was the only peptide target differentially phosphorylated within HR between Day 2 and Day 6. Overall, LR exhibit a larger number of differential phosphorylation changes following vaccination compared to HR, with the majority of these changes occurring between 2- and 6-days post-vaccination.

## 4. Discussion

In this study, we utilized a population of piglets with a wide range of antibody responses to a commercial *M. hyopneumoniae* vaccine to examine variables associated with vaccine responsiveness. Kinome analysis detected numerous phosphorylation differences within PBMCs between a discovery cohort of high and low vaccine responders with up to a 48-fold range in median serum *M. hyopneumoniae*-specific IgG titers at the time of vaccination and 6-days post-vaccination. Multiple phosphorylation differences were consistent in a validation cohort of high and low vaccine responders and these phosphorylation events were capable of differentiating high from low responders better than an untargeted kinome profile. This analysis contributes to the early molecular events occurring following vaccination between HR and LR and explores the use of peptide phosphorylation signatures which correlates with the vaccine responsiveness in piglets before and after vaccination.

Our previous investigation identified elevated concentrations of plasma IFNγ at the time of vaccination in LR compared to HR [28]. Further, LR had lower birthweight than HR despite efforts within the study design to exclude very low and very high birth weight piglets. The piglets described in our previous study were utilized as the discovery cohort in the current study, but with one subject substitution. In the current investigation, no differences in plasma cytokines and birth weight between HR and LR of the validation cohort are evident. Other studies have implicated pro-inflammatory events, such as TNFα secretion from B-cells, expression of inflammatory gene networks in PBMCs, or natural killer T-cell frequencies, to be negatively associated with vaccine-induced antibody responses in humans and mice [10,11,12,31]. As well, previous studies examining responses to typhoid vaccine found that low birth weight was considered a risk factor for impaired antibody responses within adolescent humans and infants [30,34]. The lack of effect of pro-inflammatory cytokines or birth weight on vaccine-induced IgG titers may reflect the small sample size of the validation cohort, or vaccine responses being impeded by some other factor. Alternatively, it could also be a result of the HR and LR of the validation cohort representing less extreme vaccine responsiveness than the discovery cohort, possibly suggesting that the predictive value of these physiological biomarkers is restricted to extremes of vaccine responsiveness. If the hypothesis that differences in birth weight or pro-inflammatory events can anticipate the extremes of vaccine non-responsiveness is validated, this may still be of value to livestock and human healthcare industries to identify those at risk of eliciting the weakest immune response. Ultimately, physiological factors such as plasma cytokine concentrations and body weight measurements reveal inconsistencies for discriminating HR and LR, whereas kinase-mediated signaling differences appear to be more reliable.

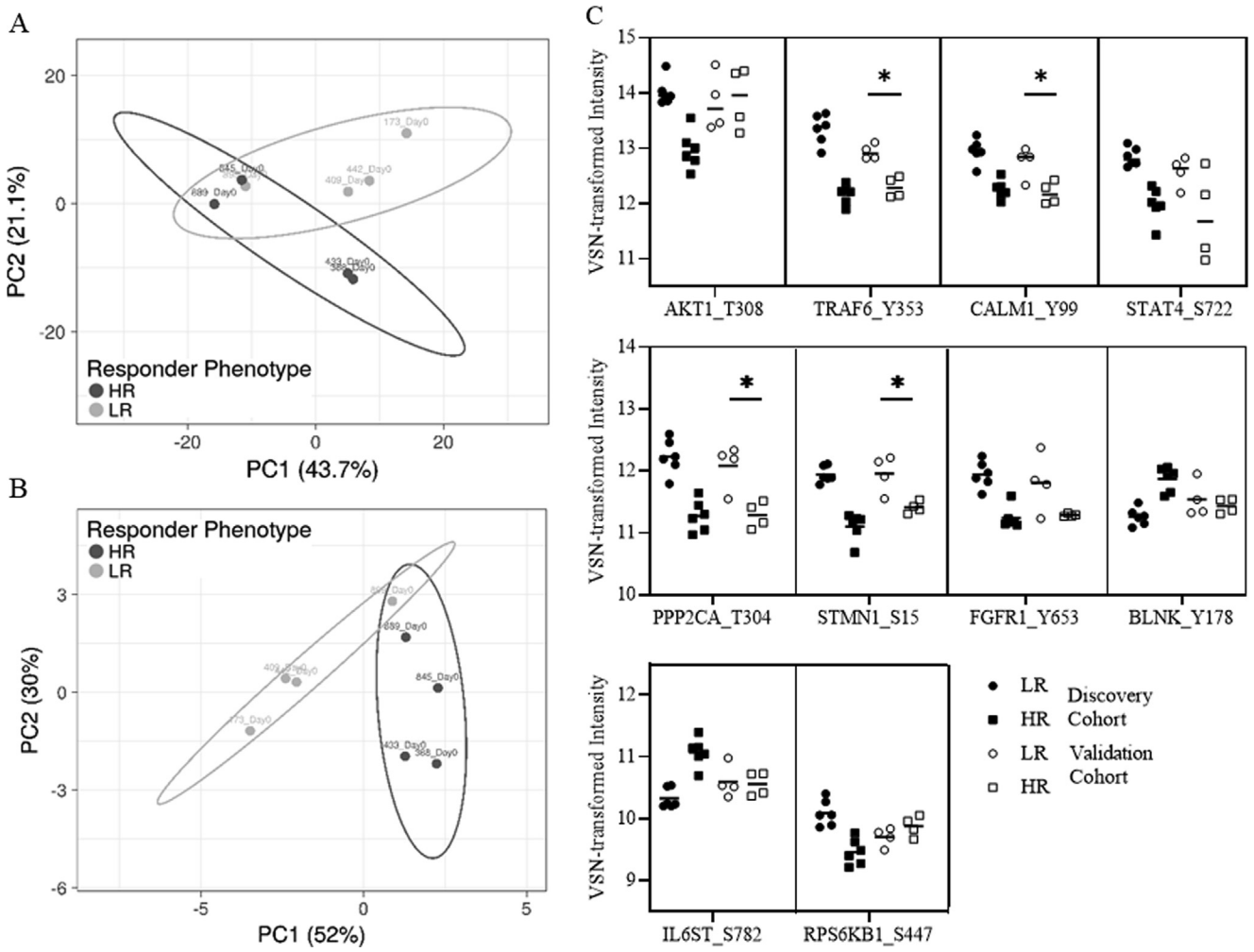


**Fig. 4. Biomarker phosphorylation events between high and low responders on Day 0 in the validation cohorts.** Principal component (PC) analysis of the high (HR; n = 4; dark grey) and low (LR; n = 4, light grey) responders within the validation cohort using **A.** 282 peptides phosphorylation events represented on the kinome array and **B.** 10 peptide phosphorylation events determined to be differentially phosphorylated between HR and LR within the discovery cohort on Day 0. PCs with the highest variance (%) are shown. Ellipses represent 95% confidence intervals. **C.** Phosphorylation intensities of the 10 differentially phosphorylated peptides on Day 0 of LR (circles) and HR (squares) within the discovery (filled shape) and validation (empty shape) cohorts. The horizontal line represents the group median. Statistical tests are only shown for comparisons between LR and HR within the validation cohort. P-values were determined using the Mann-Whitney *U* test. *p < 0.05.

Establishing the baseline phosphorylation profiles for a given tissue may be valuable in characterizing an individual's current biological state. Kinome analysis of PBMCs collected from both humans and swine have displayed individual-specific phosphorylation profiles that remained consistent over multiple weeks, suggesting individuals can possess temporally stable cell-signaling phenotypes in blood leukocytes [57]. Here, we observed numerous differences prior to vaccination at the level of phosphorylation between HR and LR within the discovery cohort, to which a subset of these events were consistent in the validation cohort. The hypothesis that host immune status can influence the response to vaccination has been an area of recent exploration [10,20,58,59]. In humans vaccinated against hepatitis B (HB) virus, non-responders (classified as having anti-HB titers < 10 IU/L 30-days post-vaccination) had greater gene expression of immune-related genes within blood and higher absolute granulocytes than responders (anti-HB > 10 IU/L) at the time of vaccination [4]. Conversely, transcriptional profiling of young individuals vaccinated against influenza virus revealed greater baseline gene expression events within high responders (classified by HAI titers 28-days post-vaccination) than low responders [20]. Our study utilizes young (<9 weeks old) piglets that may not appropriately represent a 'baseline' immune state at the time of vaccination described in other studies that use mature adults as cohorts, especially given that postnatal immune responses, hormonal factors, leukocyte populations, and cytokine production can vary within young, developing humans and livestock [6,16,24,39,40]. In swine, pre-vaccination differences in transcriptional networks were observed within PBMCs prior to *M. hyopneumoniae*-vaccination collected from high and low antibody responders defined at 118-days post-vaccination [5]. Thus, our work aligns with these previous studies supporting the hypothesis that the activity of the immune system prior to vaccination may have profound implications on the resulting immune response.

Post-vaccination biomarkers may provide insight into the biological mechanisms associated with an impaired (or unimpaired) immune response. A signature of 11 differential phosphorylation events of peptides representing proteins with immune functions and cell signaling activity were discovered between HR and LR 6-days post-vaccination. However, no phosphorylation events were discovered 2-days post-vaccination. The absence of differentially phosphorylated peptides 2-days post-vaccination may be a consequence of the strict criteria for detecting differential phosphorylation events but could be an accurate reflection of the temporal

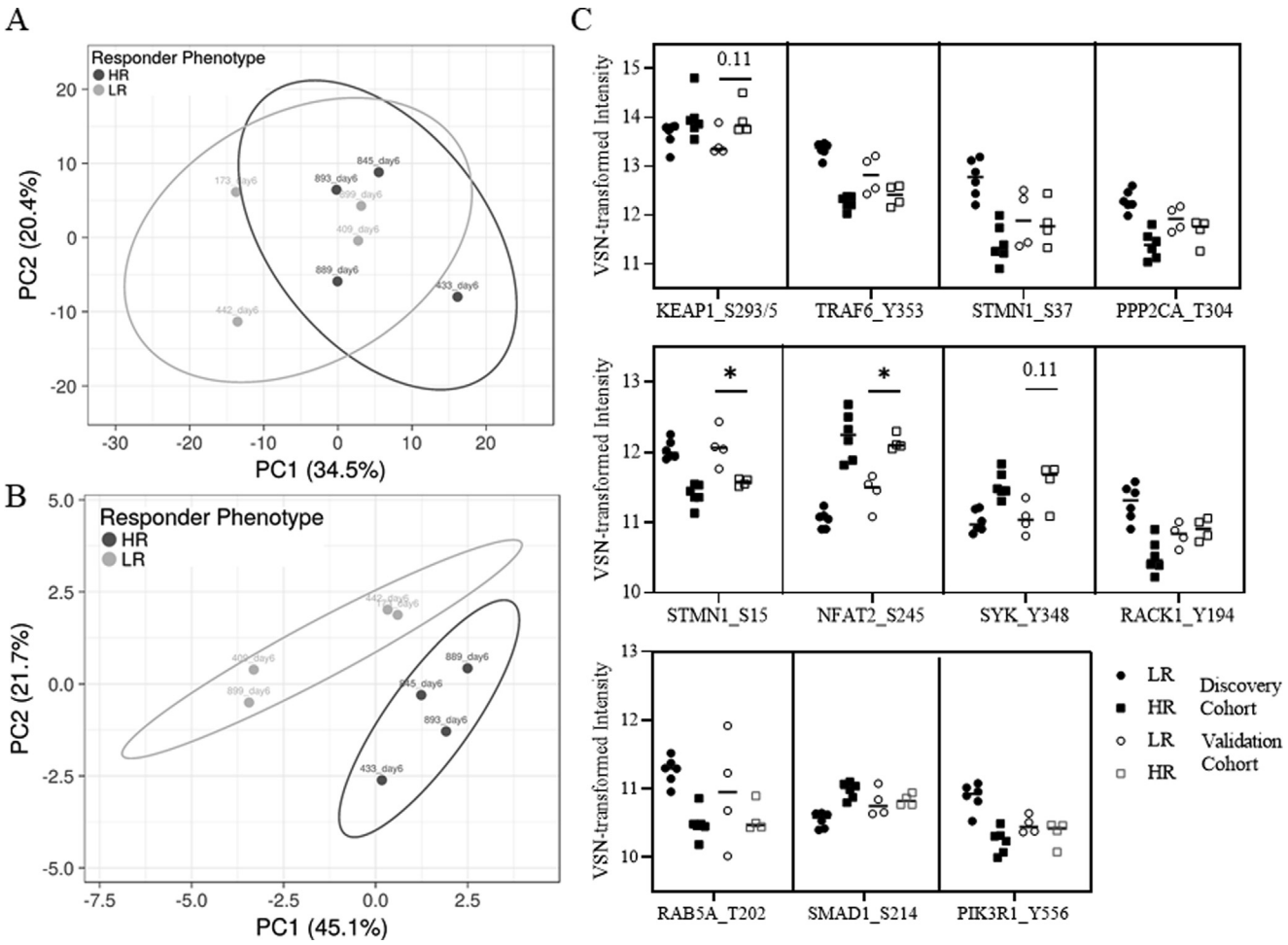


**Fig. 5. Biomarker phosphorylation events between high and low responders on Day 6 in the discovery and validation cohorts.** Principal component (PC) analysis of the high (HR; n = 4; dark grey) and low (LR; n = 4, light grey) responders within the validation cohort using **A.** 282 peptides phosphorylation events represented on the kinome array and **B.** 11 peptide phosphorylation events determined to be differentially phosphorylated between HR and LR within the discovery cohort on Day 6. PCs with the highest variance (%) are shown. Ellipses represent 95% confidence intervals. **C.** Phosphorylation intensities of the 11 differentially phosphorylated peptides on Day 6 of LR (circles) and HR (squares) within the discovery (filled shape) and validation (empty shape) cohorts. The horizontal line represents the group median. Statistical tests are only shown for comparisons between LR and HR within the validation cohort. P-values were determined using the Mann-Whitney *U* test. *p < 0.05.

signaling responses to vaccination. Comparatively, Munyaka et. al (2019) conducted a transcriptional analysis of the PBMCs of a larger sample of the HR and LR used in this study and failed to identify differentially expressed genes at the time of vaccination or at 6-days post-vaccination. However, multiple genes expressed 2-days post-vaccination were capable of discriminating animals based on *M. hyopneumoniae*-specific IgG titers [35]. None of the genes contributing to the discrimination of HR and LR in the transcriptional analysis were represented on our peptide array, nor could they be directly linked to the differentially phosphorylated targets implicated in the kinome analysis. Gene expression events involved in inflammatory and antigen presentation networks were positively correlated with vaccine-specific serum IgG responses as early as 24-hours following vaccination in pigs vaccinated with experimental *M. hyopneumoniae* vaccine formulations [29]. In pigs vaccinated against tetanus toxoid, gene transcripts involved B-cell receptor signaling were elevated within PBMCs from high anti-tetanus toxoid-antibody responders compared to low antibody responders 2–4 weeks following vaccination, yet no differences in signaling pathways were detected at the time of vaccination [1]. Given the number of peptide targets differentially phosphorylated on Day 6 in our study, these data suggest that both kinomic and transcriptomic characterizations can offer novel perspectives on the molecular changes within blood leukocytes associated with post-vaccination antibody responses in swine.

Consideration of the temporal differences in phosphorylation supports the conclusion of distinct signaling environments within the HR and LR groups between 2- and 6-days post-vaccination. Comparing the number of differentially phosphorylated events temporally changing within LR and HR reveals that LR have a greater number of changes from Day 0 to 2 (10) and Day 2 to Day 6 (21) compared to HR (0 and 1, respectively). Within this same cohort of piglets, Munyaka *et. Al* (2019) observed a greater number of gene transcripts involved in immune activation within HR at 2-days post-vaccination and a decrease in transcripts associated with immune activation at 6-days post-vaccination compared to LR [35]. At the level of kinome, we observe dissimilar trends; HR have no significant phosphorylation changes 2 and 6-days following vaccination, while LR have significant phosphorylation changes between 2 and 6-days post-vaccination. This could suggest that vaccine unresponsiveness within the population reflects the activity of cellular processes that hinder vaccine responsiveness in the LR as opposed to successful immune response mechanisms in the HR. However, this may also be reflective of certain peptide targets represented on the array that bias the detection of differential phosphorylation events to processes occurring within the LR.

**Table 2**
Phosphorylation events with differential changes between Days 0, 2, and 6 within PBMCs from low (LR) and high responders (HR).

| Target Name | Target Site | UniProt ID | Responder | Day-Day comparison | FC[a] | FDR[b] q-value |
|---|---|---|---|---|---|---|
| MDM2 | S166 | Q00987 | LR | Day 0 vs. Day 2 | −1.95 | 0.0286 |
| FOS | T232 | P01100 | LR | Day 0 vs. Day 2 | −1.52 | 0.0449 |
| IRF-3 | S402 | Q14653 | LR | Day 0 vs. Day 2 | 1.78 | 0.0449 |
| NFAT2 | S245 | O95644 | LR | Day 0 vs. Day 2 | 1.75 | 0.0449 |
| RACK1 | Y194 | P63244 | LR | Day 0 vs. Day 2 | −1.63 | 0.0449 |
| STMN1 | S37 | P16949 | LR | Day 0 vs. Day 2 | −2.76 | 0.0449 |
| SYK | Y352 | P43405 | LR | Day 0 vs. Day 2 | −1.59 | 0.0449 |
| CREB | S133 | P16220 | LR | Day 0 vs. Day 2 | −1.91 | 0.0457 |
| PIK3R1 | Y556 | P27986 | LR | Day 0 vs. Day 2 | −1.52 | 0.0457 |
| TRAF6 | Y353 | Q9Y4K3 | LR | Day 0 vs. Day 2 | −2.49 | 0.0457 |
| NFAT2 | S245 | O95644 | LR | Day 0 vs. Day 6 | −2.47 | 0.0295 |
| CALM1 | Y99 | P0DP23 | LR | Day 2 vs. Day 6 | −2.53 | 0.0136 |
| NFAT2 | S245 | O95644 | LR | Day 2 vs. Day 6 | −4.33 | 0.0136 |
| STMN1 | S15 | P16949 | LR | Day 2 vs. Day 6 | 1.66 | 0.0181 |
| BRAF1 | S579 | P15056 | LR | Day 2 vs. Day 6 | 1.70 | 0.0204 |
| FGFR1 | Y653 | P11362 | LR | Day 2 vs. Day 6 | 1.61 | 0.0272 |
| MK2 | Y415 | P16389 | LR | Day 2 vs. Day 6 | 1.77 | 0.0272 |
| PLCG2 | Y759 | P16885 | LR | Day 2 vs. Day 6 | 1.60 | 0.0272 |
| TBK1 | S172 | Q9UHD2 | LR | Day 2 vs. Day 6 | 2.30 | 0.0272 |
| TRAF6 | Y353 | Q9Y4K3 | LR | Day 2 vs. Day 6 | 2.50 | 0.0272 |
| GIT2 | Y484 | Q14161 | LR | Day 2 vs. Day 6 | 1.72 | 0.0396 |
| SMAD1 | S214 | Q15797 | LR | Day 2 vs. Day 6 | −1.38 | 0.0396 |
| AMPK1 | T174/5 | Q13131 | LR | Day 2 vs. Day 6 | 2.39 | 0.0408 |
| IRF-3 | S402 | Q14653 | LR | Day 2 vs. Day 6 | −2.26 | 0.0408 |
| MDM2 | S166 | Q00987 | LR | Day 2 vs. Day 6 | 1.58 | 0.0408 |
| NFAT3 | S676 | Q14934 | LR | Day 2 vs. Day 6 | 1.80 | 0.0408 |
| RAB5A | T202 | P20339 | LR | Day 2 vs. Day 6 | 2.01 | 0.0408 |
| SOC3 | Y221 | O14543 | LR | Day 2 vs. Day 6 | 1.66 | 0.0408 |
| STMN1 | S37 | P16949 | LR | Day 2 vs. Day 6 | 2.54 | 0.0408 |
| TAK1 | T187 | O43318 | LR | Day 2 vs. Day 6 | −1.29 | 0.0433 |
| PLCG2 | Y753 | P16885 | LR | Day 2 vs. Day 6 | 1.63 | 0.0449 |
| MAPK14 | T179 | Q16539 | LR | Day 2 vs. Day 6 | 1.85 | 0.0449 |
| CDKN1B | Y74 | P46527 | HR | Day 2 vs. Day 6 | −1.63 | 0.0295 |

[a] Fold-change (FC) is calculated as a change from the earlier Day (x) to the later Day (y) for each Day-Day comparison.
[b] False-discovery Rate (FDR) was applied to Sidak's multiple comparison tests for each set of Day-Day comparisons. FDR was set at 0.05.

Unfortunately, this study did not contain a placebo-control vaccination cohort to discern whether differential changes in phosphorylation between time-points are not a result of developmental processes within piglets which are unrelated to the vaccine response and as a result, continued investigation into the underlying mechanism of this phenotype is required.

The priorities of the current investigation were to further explore previously identified physiological biomarkers, test the capability of phosphorylation events to discriminate unknown vaccine responders, and discover post-vaccination signaling responses within PBMCs. One shortfall of this study is the lack of *in vitro* validation of the differential phosphorylation events identified between the HR and LR. Therefore, the opportunity remains to directly investigate the biological processes implicated by the phosphorylation events discovered here. To compensate, we adopted a conservative discovery approach when testing for differential phosphorylation events, including both a multiple comparison correction and a false-discovery rate correction for each time point. We utilized kinome profiles of less extreme vaccine responders as a validation cohort to test the discovered phosphorylation events. Multiple differential phosphorylation events that were tested in the validation cohort had a lower difference of intensity between HR and LR compared to the HR and LR within the discovery cohort. A similar observation was found when using phosphorylation levels as biomarkers of Varroa mite susceptibility in honeybees; baseline biomarkers discovered in subjects of the most extreme phenotype had a lower magnitude of difference when applied to independent subjects of less extreme phenotypes [47]. Additionally, while the phenotype of the validation cohort was blinded prior to kinome analyses, all animals originated from the same facility and samples were processed on the same day. Systemic factors due to animal handling or vaccination administration may have contributed to the variation in vaccine-induced antibody responses. Therefore, all differences detected between the vaccine responders of this study must be replicated in an independent population of vaccinated swine. As well, kinome analysis was performed only on vaccine responders with the highest and lowest serum IgG responses. The phosphorylation events described here may not linearly correlate with vaccine-induced serum IgG responses across the entire population, and future studies should consider incorporating vaccine responders of median/average responses for evaluating predictive capacities [20,53].

In conclusion, phosphorylation events are novel biomarkers of vaccine responsiveness that are more robust in discriminating HR and LR in this population than some physiological markers. Vaccine responsiveness biomarkers could also provide a tool to enable more efficacious vaccination programs through the prescreening of individuals to identify anticipated non-responders. Individuals predicted to have impaired responses to the vaccine could be candidates for alternative regimens such as revaccination, novel vaccination scheduling, or limiting social interaction with the remainder of the population until effective vaccination can be achieved. Additionally, kinome analysis presents as a technique sensitive for delineating the complex phenotypes within heterogeneous species that may complement other systems biology approaches. A future objective of this research includes using differential phosphorylation signatures for predicting vaccine responses in an independent population of piglets and testing the robustness of prediction using other vaccine antigens. As well, future trials should incorporate a metric of T-cell responses as it is unknown whether these signatures only correlate with antibody responses alone. Ultimately, these findings present a new

perspective of the molecular events specifically associated with vaccine responses within a species both critical to the livestock industry and highly relevant to human immunology [18,33]. Particularly in livestock species, anticipating vaccine responsiveness would be of considerable value and importance for implementing more effective vaccination programs.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Acknowledgments

This research was enabled by individual Discovery Grants from the Natural Sciences and Engineering Research Council of Canada (NSERC) to SN [RGPIN 05690-2018]. VIDO receives operational funding from the Government of Saskatchewan through Innovation Saskatchewan and the Ministry of Agriculture and from the Canada Foundation for Innovation through the Major Science Initiatives for its CL3 facility (InterVac).

Published as VIDO-InterVac manuscript series no. 948

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jvacx.2022.100167.

## References

[1] Adler M, Murani E, Ponsuksili S, Wimmers K. PBMC transcriptomic responses to primary and secondary vaccination differ due to divergent lean growth and antibody titers in a pig model. Physiol Genomics 2015;47(10):470–8.
[2] Arsenault R, Griebel P, Napper S. Peptide Arrays for Kinome Analysis: New Opportunities and Remaining Challenges. Proteomics 2011;11(24):4595–609.
[3] Arsenault RJ, Li Y, Maattanen P, Scruten E, Doig K, Potter A, et al. Altered toll-like receptor 9 signaling in *Mycobacterium avium* subsp. *paratuberculosis*-infected bovine monocytes reveals potential therapeutic targets. Infect Immun 2013;81(1):226–37.
[4] Bartholomeus E, De Neuter N, Meysman P, Suls A, Keersmaekers N, Elias G, et al. Transcriptome profiling in blood before and after hepatitis B vaccination shows significant differences in gene expression between responders and non-responders. Vaccine 2018;36(42):6282–9.
[5] Blanc F, Maroilley T, Revilla M, Lemonnier G, Leplat J-J, Billon Y, et al. Influence of genetics and the pre-vaccination blood transcriptome on the variability of antibody levels after vaccination against *Mycoplasma hyopneumoniae* in pigs. Genet Sel Evol 2021;53(1). https://doi.org/10.1186/s12711-021-00614-5.
[6] Chase CCL, Hurley DJ, Reber AJ. Neonatal Immune Development in the Calf and Its Impact on Vaccine Response. Vet. Clin North Am - Food Anim Pract 2008;24(1):87–104.
[7] Chen Y, Stookey J, Arsenault R, Scruten E, Griebel P, Napper S. Investigation of the physiological, behavioral, and biochemical responses of cattle to restraint stress. J Anim Sci 2016;94:3240–54.
[8] Djordjevic SP, Eamens GJ, Romalis LF, Nicholls PJ, Taylor V, Chin J. Serum and mucosal antibody responses and protection in pigs vaccinated against *Mycoplasma hyopneumoniae* with vaccines containing a denatured membrane antigen pool and adjuvant. Aust Vet J 1997;75(7):504–11.
[9] Facciuolo A, Denomy C, Lipsit S, Kusalik A, Napper S. From Beef to Bees: High-Throughput Kinome Analysis to Understand Host Responses of Livestock Species to Infectious Diseases and Industry-Associated Stress. Front Immunol 2020;11. https://doi.org/10.3389/fimmu.2020.00765.
[10] Fourati S, Cristescu R, Loboda A, Talla A, Filali A, Railkar R, et al. Pre-vaccination inflammation and B-cell signalling predict age-related hyporesponse to hepatitis B vaccination. Nat Commun 2016;7(1). https://doi.org/10.1038/ncomms10369.
[11] Frasca D, Blomberg BB. Inflammaging decreases adaptive and innate immune responses in mice and humans. Biogerontology 2016;17(1):7–19.
[12] Frasca D, Diaz A, Romero M, Landin AM, Blomberg BB. High TNF-α levels in resting B cells negatively correlate with their response. Exp Gerontol 2014;54:116–22.
[13] Furman D, Jojic V, Kidd B, Shen-Orr S, Price J, Jarrell J, et al. Apoptosis and other immune biomarkers predict influenza vaccine responsiveness. Mol Syst Biol 2013;9(1):659. https://doi.org/10.1038/msb.2013.15.
[14] Gaucher D, Therrien René, Kettaf N, Angermann BR, Boucher Geneviève, Filali-Mouhim A, et al. Yellow fever vaccine induces integrated multilineage and polyfunctional immune responses. J Exp Med 2008;205(13):3119–31.
[15] Gonzalez-Dias P, Lee EK, Sorgi S, de Lima DS, Urbanski AH, Silveira EL, et al. Methods for predicting vaccine immunogenicity and reactogenicity. Hum Vaccines Immunother 2020;16(2):269–76.
[16] de Groot J, Kruijt L, Scholten JW, Boersma WJA, Buist WG, Engel B, et al. Age, gender and litter-related variation in T-lymphocyte cytokine production in young pigs. Immunology 2005;115(4):495–505.
[17] Hagan T, Cortese M, Rouphael N, Boudreau C, Linde C, Maddur MS, et al. Antibiotics-Driven Gut Microbiome Perturbation Alters Immunity to Vaccines in Humans. Cell 2019;178(6):1313–1328.e13.
[18] Hein WR, Griebel PJ. A road less travelled: Large animal models in immunological research. Nat Rev Immunol 2003;3(1):79–84.
[19] Heininger U, Bachtiar NS, Bahri P, Dana A, Dodoo A, Gidudu J, et al. The concept of vaccination failure. Vaccine 2012;30(7):1265–8.
[20] HIPC-CHI Signatures Project Team, Consortium H.-I. Multicohort analysis reveals baseline transcriptional predictors of influenza vaccination responses. Sci Immunol 2017;2.
[21] de Jong SE, Olin A, Pulendran B. The Impact of the Microbiome on Immunity to Vaccination in Humans. Cell Host Microbe 2020;28(2):169–79.
[22] Jouneau L, Lefebvre DJ, Costa F, Romey A, Blaise-Boisseau S, Relmy A, et al. The antibody response induced FMDV vaccines in sheep correlates with early transcriptomic responses in blood. npj Vaccines 2020;5(1). https://doi.org/10.1038/s41541-019-0151-3.
[23] Kindrachuk J, Arsenault R, Kusalik A, Kindrachuk KN, Trost B, Napper S, et al. Systems Kinomics Demonstrates Congo Basin Monkeypox Virus Infection Selectively Modulates Host Cell Signaling Responses as Compared to West African Monkeypox Virus M111.015701. Mol Cell Proteomics 2012;11(6). https://doi.org/10.1074/mcp.M111.015701.
[24] Kollmann TR, Marchant A. Towards Predicting Protective Vaccine Responses in the Very Young. Trends Immunol 2016;37(8):523–34.
[25] Kotliarov Y, Sparks R, Martins AJ, Mulè MP, Lu Y, Goswami M, et al. Broad immune activation underlies shared set point signatures for vaccine responsiveness in healthy individuals and disease activity in patients with lupus. Nat Med 2020;26(4):618–29.
[26] Lambert ND, Ovsyannikova IG, Pankratz VS, Jacobson RM, Poland GA. Understanding the immune response to seasonal influenza vaccination in older adults: a systems biology approach. Expert Rev Vaccines 2012;11(8):985–94.
[27] Li S, Sullivan NL, Rouphael N, Yu T, Banton S, Maddur MS, et al. Metabolic Phenotypes of Response to Vaccination in Humans. Cell 2017;169(5):862–877.e17.
[28] Lipsit SWL, Wilkinson J, Scruten E, Facciuolo A, Denomy C, Griebel PJ, et al. Kinome profiling of peripheral blood mononuclear cells collected prior to vaccination reveals biomarkers and potential mechanisms of vaccine unresponsiveness in pigs. Sci Rep 2020;10:11546.
[29] Matthijs AMF, Auray G, Jakob V, García-Nicolás O, Braun RO, Keller I, et al. Systems Immunology Characterization of Novel Vaccine Formulations for *Mycoplasma hyopneumoniae* Bacterins. Front Immunol 2019;10. https://doi.org/10.3389/fimmu.2019.0108710.3389/fimmu.2019.01087.s00110.3389/fimmu.2019.01087.s002.
[30] McDade TW, Beck MA, Kuzawa C, Adair LS. Prenatal undernutrition, postnatal environments, and antibody response to vaccination in adolescence. Am J Clin Nutr 2001;74(4):543–8.
[31] Mcdade TW, Adair L, Feranil AB, Kuzawa C. Positive antibody response to vaccination in adolescence predicts lower C-reactive protein concentration in young adulthood in the Philippines. Am J Hum Biol 2011;23(3):313–8.
[32] Metsalu T, Vilo J. ClustVis: A web tool for visualizing clustering of multivariate data using Principal Component Analysis and heatmap. Nucleic Acids Res 2015;43(W1):W566–70.
[33] Meurens F, Summerfield A, Nauwynck H, Saif L, Gerdts V. The pig: a model for human infectious diseases. Trends Microbiol 2012;20(1):50–7.
[34] Moore SE, Jalil F, Ashraf R, Chen Szu S, Prentice AM, Hanson LÅ. Birth weight predicts response to vaccination in adults born in an urban slum in Lahore. Pakistan Am J Clin Nutr 2004;80(2):453–9.
[35] Munyaka PM, Kommadath A, Fouhse J, Wilkinson J, Diether N, Stothard P, et al. Characterization of whole blood transcriptome and early-life fecal microbiota in high and low responder pigs before, and after vaccination for *Mycoplasma hyopneumoniae*. Vaccine 2019;37(13):1743–55.
[36] Nakaya HI, Wrammert J, Lee EK, Racioppi L, Marie-Kunze S, Haining WN, et al. Systems biology of vaccination for seasonal influenza in humans. Nat Immunol 2011;12(8):786–95.
[37] Nakaya HI, Clutterbuck E, Kazmin D, Wang L, Cortese M, Bosinger SE, et al. Systems biology of immunity to MF59-adjuvanted versus nonadjuvanted trivalent seasonal influenza vaccines in early childhood. Proc Natl Acad Sci USA 2016;113(7):1853–8.
[38] Napper S, Dadgar S, Arsenault RJ, Trost B, Scruten E, Kusalik A, et al. Induction of tissue- and stressor-specific kinomic responses in chickens exposed to hot and cold stresses. Poult Sci 2015;94(6):1333–45.
[39] Nguyen TV, Yuan L, Azevedo MSP, Jeong K-I, Gonzalez A-M, Saif LJ. Transfer of maternal cytokines to suckling piglets: In vivo and in vitro models with implications for immunomodulation of neonatal immunity. Vet Immunol Immunopathol 2007;117(3-4):236–48.
[40] Olin A, Henckel E, Chen Y, Lakshmikanth T, Pou C, Mikes J, et al. Stereotypic Immune System Development in Newborn Children. Cell 2018;174(5):1277–1292.e14.

[41] Osterholm MT, Kelley NS, Sommer A, Belongia EA. Efficacy and effectiveness of influenza vaccines: A systematic review and meta-analysis. Lancet Infect Dis 2012;12(1):36–44.
[42] Ovsyannikova IG, Haralambieva IH, Vierkant RA, Pankratz VS, Jacobson RM, Poland GA. The role of polymorphisms in Toll-like receptors and their associated intracellular signaling genes in measles vaccine immunity. Hum Genet 2011;130:547–61.
[43] Park H-L, Shim S-H, Lee E-Y, Cho W, Park S, Jeon H-J, et al. Obesity-induced chronic inflammation is associated with the reduced efficacy of influenza vaccine. Hum Vaccin Immunother 2014;10(5):1181–6.
[44] Posteraro B, Pastorino R, Di Giannantonio P, Ianuale C, Amore R, Ricciardi W, et al. The link between genetic variation and variability in vaccine responses: Systematic review and meta-analyses. Vaccine 2014;32(15):1661–9.
[45] Pulendran B, Li S, Nakaya HI. Systems vaccinology. Immunity 2010;33 (4):516–29.
[46] Robertson AJ, Trost B, Scruten E, Robertson T, Mostajeran M, Connor W, et al. Identification of developmentally-specific kinotypes and mechanisms of Varroa mite resistance through whole-organism, kinome analysis of honeybee. Front Genet 2014;5. https://doi.org/10.3389/fgene.2014.00139.
[47] Robertson AJ, Scruten E, Mostajeran M, Robertson T, Denomy C, Hogan D, et al. Kinome Analysis of Honeybee (*Apis mellifera L.*) Dark-Eyed Pupae Identifies Biomarkers and Mechanisms of Tolerance to Varroa Mite Infestation. Sci Rep 2020;10(1). https://doi.org/10.1038/s41598-020-58927-2.
[48] Shannon CP, Blimkie TM, Ben-Othman R, Gladish N, Amenyogbe N, Drissler S, et al. Multi-Omic Data Integration Allows Baseline Immune Signatures to Predict Hepatitis B Vaccine Response in a Small Cohort. Front Immunol 2020;11. https://doi.org/10.3389/fimmu.2020.57880110.3389/fimmu.2020.578801.s00110.3389/fimmu.2020.578801.s00210.3389/fimmu.2020.578801.s00310.3389/fimmu.2020.578801.s00410.3389/fimmu.2020.578801.s00510.3389/fimmu.2020.578801.s00610.3389/fimmu.2020.578801.s00710.3389/fimmu.2020.578801.s00810.3389/fimmu.2020.578801.s00910.3389/fimmu.2020.578801.s01010.3389/fimmu.2020.578801.s01110.3389/fimmu.2020.578801.s01210.3389/fimmu.2020.578801.s01310.3389/fimmu.2020.578801.s01410.3389/fimmu.2020.578801.s015.
[49] Sheridan PA, Paich HA, Handy J, Karlsson EA, Hudgens MG, Sammon AB, et al. Obesity is associated with impaired immune response to influenza vaccination in humans. Int J Obes 2012;36(8):1072–7.
[50] Sjogren MH. Prevention of hepatitis B in nonresponders to initial hepatitis B virus vaccination. Am J Med 2005;118(10):34–9.
[51] Smith SE, Mellor P, Ward AK, Kendall S, McDonald M, Vizeacoumar FS, et al. Molecular characterization of breast cancer cell lines through multiple omic approaches. Breast Cancer Res 2017;19(1). https://doi.org/10.1186/s13058-017-0855-0.
[52] Thacker EL, Thacker BJ, Boettcher TB, Jayappa H. Comparison of antibody production, lymphocyte stimulation, and protection induced by four commercial *Mycoplasma hyopneumoniae* bacterins. J. Swine Health Prod. 1998;6:107–12.
[53] Thompson-Crispi KA, Miglior F, Mallard BA. Incidence rates of clinical mastitis among Canadian holsteins classified as high, average, or low immune responders. Clin Vaccine Immunol 2013;20(1):106–12.
[54] Thompson-Crispi KA, Sargolzaei M, Ventura R, Abo-Ismail M, Miglior F, Schenkel F, et al. A genome-wide association study of immune response traits in Canadian Holstein cattle. BMC Genomics 2014;15(1):559. https://doi.org/10.1186/1471-2164-15-559.
[55] Tomic A, Tomic I, Rosenberg-Hasson Y, Dekker CL, Maecker HT, Davis MM. SIMON, an Automated Machine Learning System, Reveals Immune Signatures of Influenza Vaccine Responses. J Immunol 2019;203(3):749–59.
[56] Trost B, Kindrachuk J, Määttänen P, Napper S, Kusalik A, Xue Yu. PIIKA 2: an expanded, web-based platform for analysis of kinome microarray data. PLoS ONE 2013;8(11):e80837. https://doi.org/10.1371/journal.pone.008083710.1371/journal.pone.0080837.g00110.1371/journal.pone.0080837.g00210.1371/journal.pone.0080837.g00310.1371/journal.pone.0080837.g00410.1371/journal.pone.0080837.g00510.1371/journal.pone.0080837.g00610.1371/journal.pone.0080837.g00710.1371/journal.pone.0080837.g00810.1371/journal.pone.0080837.g00910.1371/journal.pone.0080837.t00110.1371/journal.pone.0080837.s00110.1371/journal.pone.0080837.s00210.1371/journal.pone.0080837.s00310.1371/journal.pone.0080837.s00410.1371/journal.pone.0080837.s005.
[57] Trost B, Kindrachuk J, Scruten E, Griebel P, Kusalik A, Napper S. Kinotypes: stable species- and individual-specific profiles of cellular kinase activity. BMC Genomics 2013;14:854.
[58] Tsang J, Schwartzberg P, Kotliarov Y, Biancotto A, Xie Z, Germain R, et al. Global analyses of human immune variation reveal baseline predictors of postvaccination responses. Cell 2014;157(2):499–513.
[59] Tsang JS, Dobaño C, VanDamme P, Moncunill G, Marchant A, Othman RB, et al. Improving Vaccine-Induced Immunity: Can Baseline Predict Outcome? Trends Immunol 2020;41:457–65.
[60] Van Wyk B, Snider M, Scruten E, van Drunen Littel-van den Hurk S, Napper S. Induction of functional interferon alpha and gamma responses during acute infection of cattle with non-cytopathic bovine viral diarrhea virus. Vet Microbiol 2016;195:104–14.
[61] Zhang Lu, Wang W, Wang S. Effect of Vaccine Administration Modality on Immunogenicity and Efficacy. Expert Rev Vaccines 2015;14(11):1509–23.