Supplemental Digital Content 6:

Principal Component Analysis (PCA)

The PCA analysis was able to comprise the variables into 5 components, explaining 95% of the data variations. PC1 corresponds to the overall immune activity with all cytokines loading in the same direction. This component explains 64% of the variation. PC2 shows a clear distinction between to the bacteria used for immune stimulation and when combined with PC1, 79% of the immune profile variation is captured. Figure E-2 pictures the loading plot of the dominating sources of variation, PC1 and PC2.

The overall immune activity (PC1) was not associated with incidence of LRI ($p= 0.40$), meaning that the general tendency to respond with high or low levels of cytokines is not associated to susceptibility to LRI. PC2 shows a clear distinction between to the bacteria used for immune stimulation, and this component was significantly associated to incidence of LRI ($p=0.02$), meaning that the three bacteria tend to induce different stimulation signals.

Beyond PC1 and PC2 are the components that capture the subtle variations, i.e. deviations from the general immune pattern exhibited by the first two components. There were two components significantly associated to incidence of LRI, namely PC2 ($p= 0.02$) and PC5 ($p= 0.005$), explaining 4.8% of the variation. Figure E-3 shows PC2 and PC5 in combination. This plot shows that when eliminate other variations in the data not relevant for incidence of LRI, then TNF-α, IL-5 and IFN-γ are the most important in for both components in describing variation in incidence of LRI (long distance from zero on both x- and y-axis), but other cytokines are also relevant.

The link between the two components is not obvious. To pursue this link we plotted the mean response of each cytokine for the three bacteria against each other. Figure E-4 shows the results for the TNF-α stimulations. What we see in figure E-4 is that for TNF-α the mean response to HI is usually very similar to the mean response to MC stimulation (the slope of the regression line is close to 1). However, the SP-response tends to differ from the two other stimulations, and healthy children have lower TNF-α levels in response to SP compared to their response to HI/MC (the slope is below 1). But the more the SP-response is skewed towards the HI/MC response (increasing slope of the line), the more likely is the child of having LRI. Similar pictures are drawn for IFN-γ

(fig.E-5) and for IL-5 (fig E-6), although for IL-5 it is the ratio between MC and HI simulations that is important.

The interpretation of these results is indeed very complex, and the understanding of biological mechanisms is even more. Therefore, we are reluctant to draw further conclusions on a cytokine or bacteria specific level and further details on the analysis is not included here.

Figure E-2: Loading plot, PC1 versus PC2
Including the percentage of variation described by the component and the p-value on the association to no. of LRI.

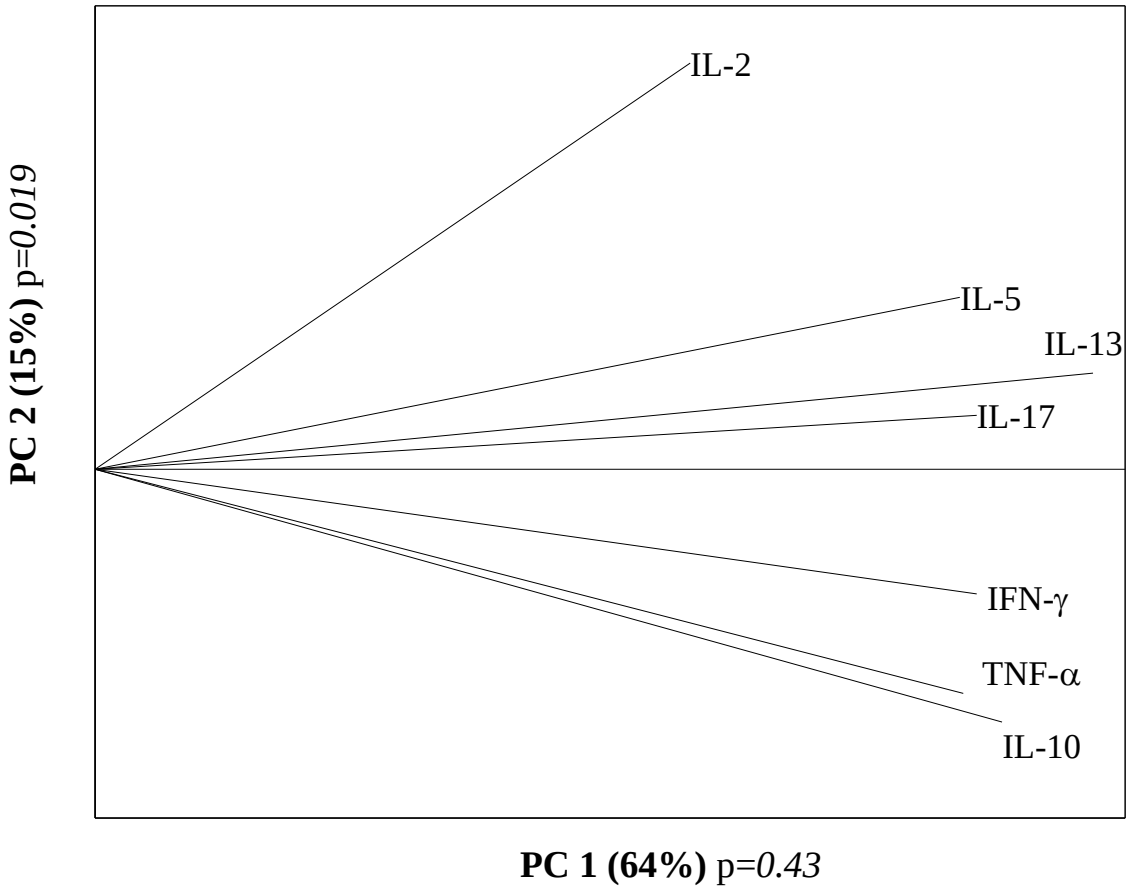

Figure E-3: Loading plot PC2 versus PC5.
Including the percentage of variation described by the component and the p-value on the association to no. of LRI.

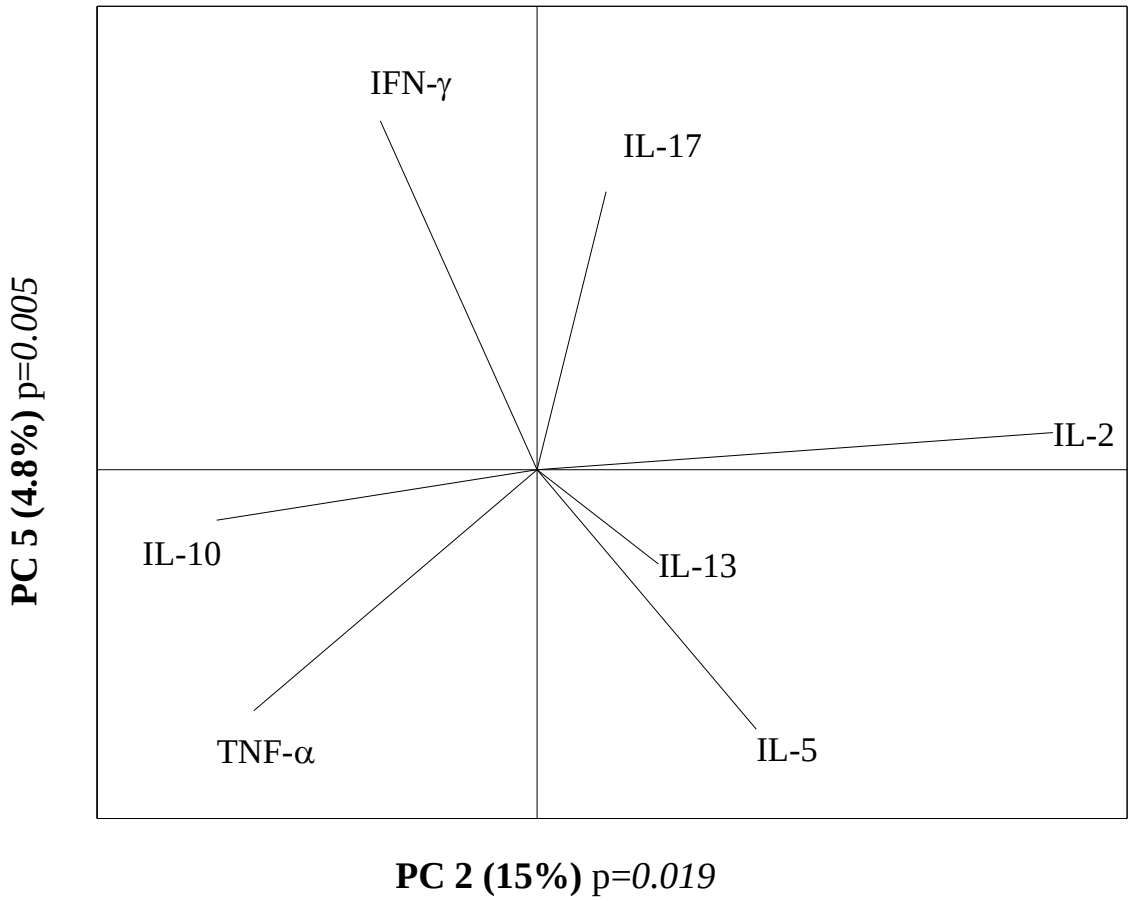

Figure E-4: Individual TNF-α levels (square root transformed after subtraction of control) for each child with the 3 bacterial stimulations plotted against each other, stratified by incidence of LRI.

■: No. of LRI = 0 ■: No. of LRI = 1 ■: No. of LRI > 1
HI: *H. influenzae*, MC: *M. catarrhalis*, SP: *S. pneumoniae*.

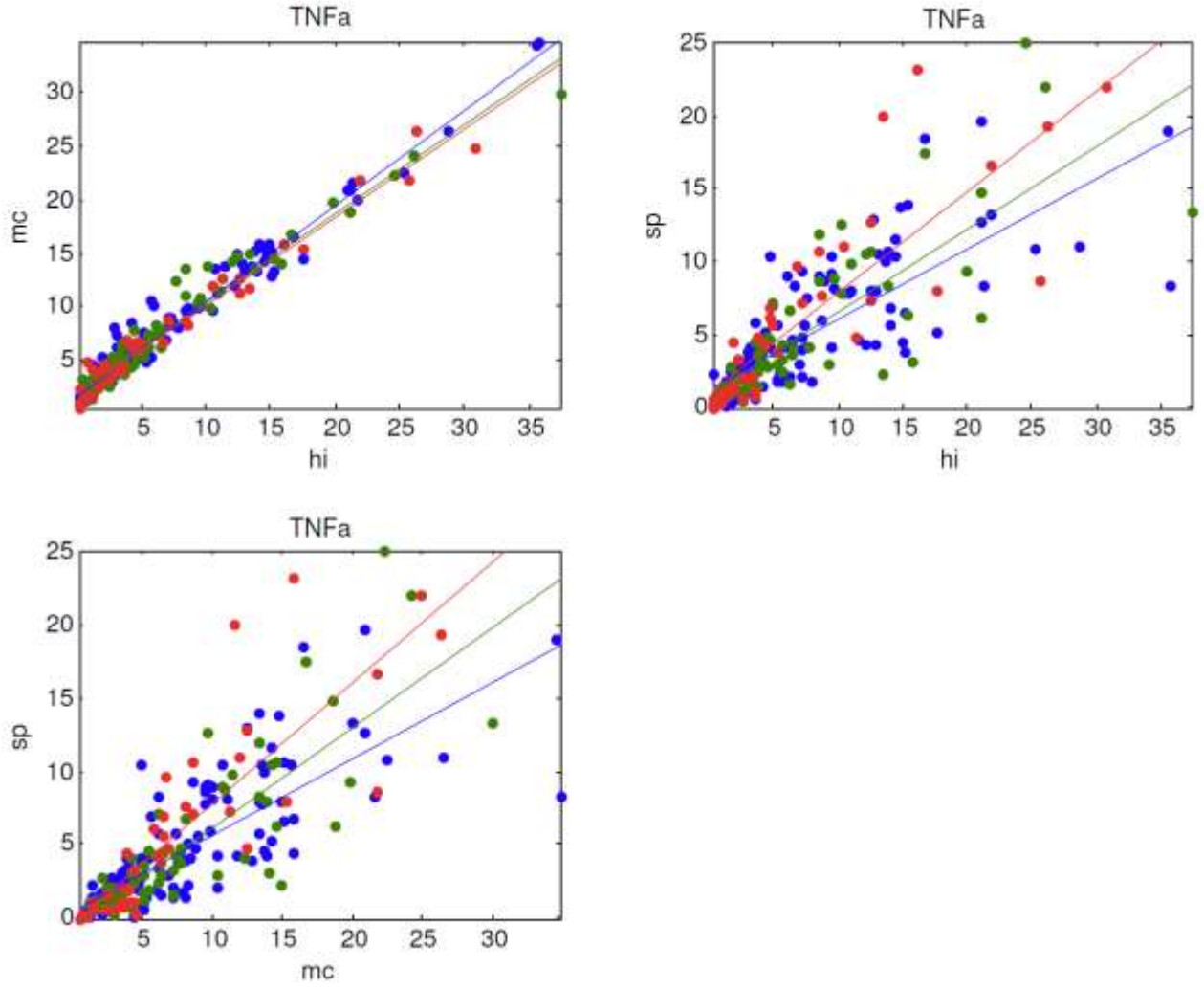

Figure E-5: Individual IFN-γ levels (square root transformed after subtraction of control) for each child with the 3 bacterial stimulations plotted against each other, stratified by incidence of LRI.

■: No. of LRI = 0 ■: No. of LRI = 1 ■: No. of LRI > 1
HI: *H. influenzae*, MC: *M. catarrhalis*, SP: *S. pneumoniae*.

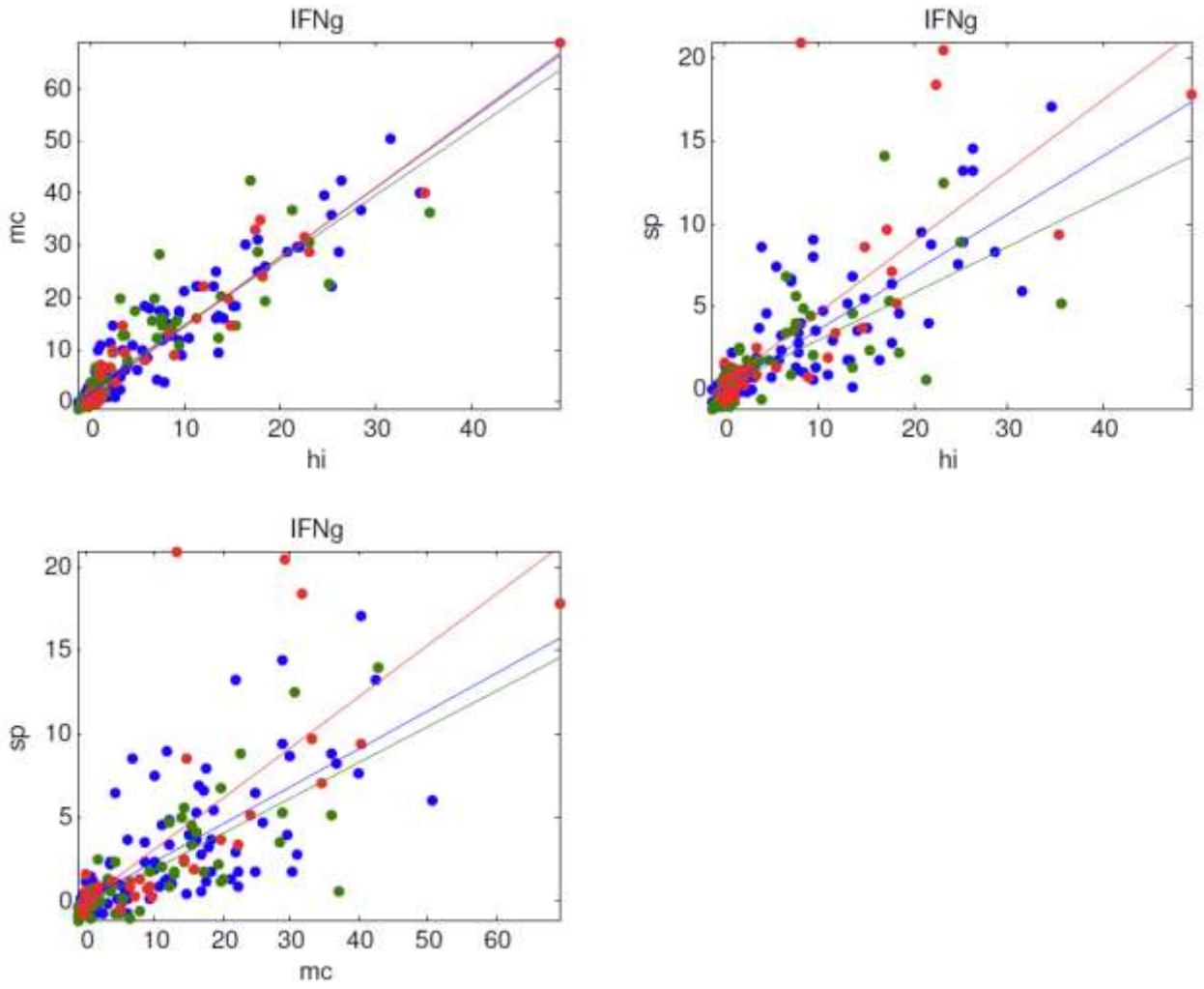

Figure E-6: Individual IL-5 levels (square root transformed after subtraction of control) for each child with the 3 bacterial stimulations plotted against each other, stratified by incidence of LRI.

■: No. of LRI = 0 ■: No. of LRI = 1 ■: No. of LRI > 1
HI: *H. influenzae*, MC: *M. catarrhalis*, SP: *S. pneumoniae*.