International LIVE vaccinology

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Impact of Inflammation on Responses to Vaccination in the Elderly

Master de Sciences, Technologies, Santé, Mention Biologie Moléculaire et Cellulaire
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Master Thesis

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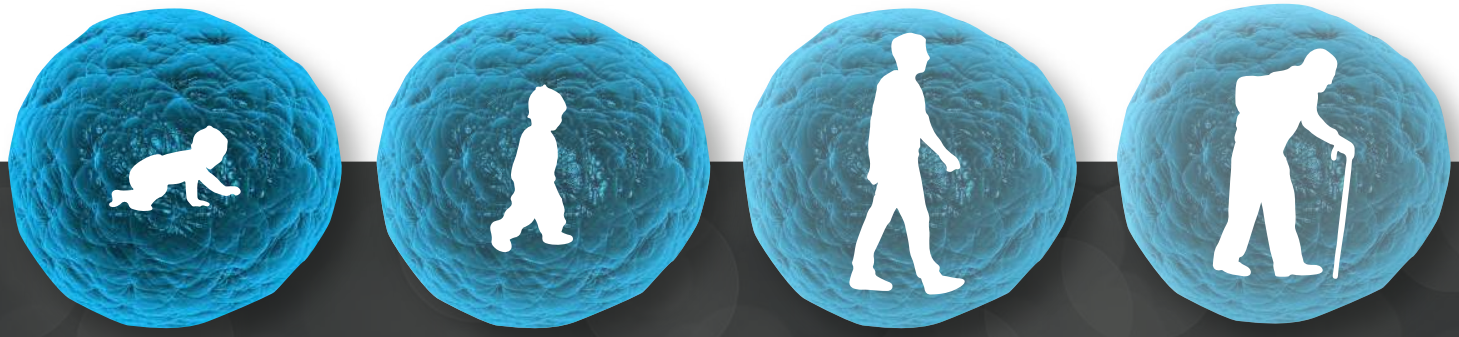

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Summary

Vaccine development for the elderly has proven to be a challenge given the diminished and highly heterogenous immune response observed in this population. This hyporesponsiveness results from a series of changes that occur to the ageing immune system, among which inflammaging has been shown to be particularly detrimental. To gain further insight on how this chronic inflammation affects the response to immunization, 25 inflammatory-related biomarkers were measured in serum from 316 subjects of different ages from the VITAL cohort. Concentration changes of each biomarker were found to follow one of three trajectories describing how inflammation increases according to age. Some biomarkers such as IL-1Ra, neopterin, YKL-40, and IL-6, which were shown to moderately correlate with age, and were also found to contribute the most at explaining the variability in the data. Using these 25 biomarkers, two machine learning models to predict vaccine responsiveness were developed. The prediction accuracy rate was higher for the support vector machine-based model (73%) compared to that of the lasso logistic regression (67%). Although these models require further validation, this proof-of-concept shows that inflammation biomarkers at baseline are indeed able to predict the quality of the immune response. Finally, a spectral cytometry panel is developed that allows for the characterization of multiple immune parameters on T cells *in-vitro.* This panel is expected to be used in the future to study the differences on the immune response to the SARS-CoV-2 vaccine between adult and elderly subjects in the VITAL cohort.

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List of Abbreviations

AID	*Activation-induced cytidine deaminase*
APC	*Antigen presenting cell*
Angpt-2	*Angiopoietin 2*
BMI	*Body mass index*
ConA	*Concanavalin A*
CMV	*Cytomegalovirus*
CRP	*C reactive protein*
DAMP	*Damage-associated molecular patterns*
ERK	*Extracellular signal-regulated kinase*
HAI	*Haemagglutination inhibition*
HLA	*Human leukocyte antigen*
iFABP2	*Intestinal fatty acid binding protein 2*
IFN- γ	*Interferon-*γ
IMP	*Immunosenescence phenotype*
LAIV	*Live attenuated Influenza vaccine*
MHC	*Major histocompatibility complex*
NET	*Neutrophil extracellular trap*
NGS	*Next generation sequencing*
PBMC	*Peripheral blood mononuclear cell*
PR3	*Proteinase 3*
PTX3	*Pentraxin-related protein*
QIV	*Quadrivalent inactivated Influenza vaccine*
ROS	*Reactive oxygen species*
SASP	*Senescent-associated secretory phenotype*
SEB	*Streptococcus enterotoxin B*
SVM	*Support vector machine*
TCR	*T cell receptor*
TIV	*Trivalent inactivated Influenza vaccine*
TLR	*Toll-like receptor*
TNF-α	*Tumour necrosis factor -* α
YKL-40	*Chitinase-3-like protein 1*

1. Introduction:

In the last hundred years the life expectancy at birth around the world has significantly increased to the current global average of 70 years. This trend has contributed to the increase in the number of people 60 years or older which has more than doubled since 1980, and which it is estimated to reach 2.1 billion people by 2050 (United Nations. Department of Economic and Social Affairs, 2017). Unfortunately, the healthy life expectancy has not grown at the same pace, meaning that the gap in years spent in ill-health has been slowly increasing despite our progress in the medical sciences. (World Health Organization, 2020). Interest for different interventions targeting the health needs of the elderly population has therefore been growing in the last decade. Among them, significant progress has been achieved in the field of vaccination, where new formulations, adjuvants, and technologies have been developed to address diseases such as influenza, pneumococcal disease, and shingles; diseases which disproportionally affect this segment of the population (Pinti et al., 2016).

Despite these advances, developing vaccines for the elderly has proven to be challenging given the diminished and heterogenous response that is observed in this population (Fulop et al., 2020). This hyporesponsiveness is in great part the result of a series of changes in the immune system associated with ageing, a phenomenon known as immunosenescence. The present literature revision aims at reviewing what are some of the most significant changes of the ageing immune system, and how, in the past decade, the systems biology and machine learning approaches have been used to gain a better insight of the immunosenescence process and its relation to vaccine responsiveness.

Although research on the relation between ageing and the changes in the immune system was already taking place in the 1960's, the term "immunosenescence" was not properly introduced in the literature until the late 1970's by Takashi Makinodan to refer to the predisposition to disease that resulted from the decline of the immune functions with age (1977). Nonetheless, as pointed out by Pawelec (2017), immunosenescence as a term remains loosely defined, and this has led to some overlap with other concepts such as replicative senescence. He suggests immunosenescence to be defined as a state of "*robust measures of immune parameters (biomarkers) that are different in younger and older individuals and which have been associated with a clearly detrimental clinical outcome*." This interpretation is probably the most frequently used by the immunologists in the field, and it emphasises one aspect that has been particularly controversial. Wanting to restrict immunosenescence to those changes causing detrimental clinical outcomes only, arises from some critiques that have accused the field to have an ageist bias (Fulop et al., 2020; Pawelec, 2018; Pawelec et al., 2020). For instance, special attention has been given to changes in the repertoire, which are generally considered as detrimental to the quality of the adaptive immune response, but which has also been argued to be consistent with an evolutionary adaptation that aims at conferring greater protection against the local dangers (Fulop et al., 2020). In fact, recent evidence also suggests that preferential proliferation of $CD8^+$ T cells in humans is a mechanisms to preserve naïve T cells for when most needed (Mayya et al., 2019). Other examples include the increased immunosuppression seen in the elderly, either by an increases number of Tregs, or by a higher expression of immunomodulatory molecules such as PD-1. While these changes in an adult individual might point at a hampered immune response, in the elderly these mechanisms are probably adaptations that regulate inflammaging (Fulop et al., 2020), a low-grade chronic inflammation state that will be discussed latter. Ultimately, when interpreting data on the age-associated immune changes and their effects, it is important to keep in mind these changes are

not implicitly unfavourable, with some of them potentially being adaptations to an overall changing individual.

1.1 Characteristics of the ageing immune system

One of the main challenges in studying immunosenescence is the fact that it results from several immune changes that influence one another. To facilitate their study, these changes can be grouped six major processes which describe the hallmark features of the immunosenescence phenomenon (*Figure 1*). These hallmarks are: inflammaging, memory inflation, defective cell migration, myeloid-biased haematopoiesis, hampered affinity maturation, and accumulation of senescent lymphocytes. However, each of these six also results from a series of discreet changes that occur to individual immune cell subsets as follows:

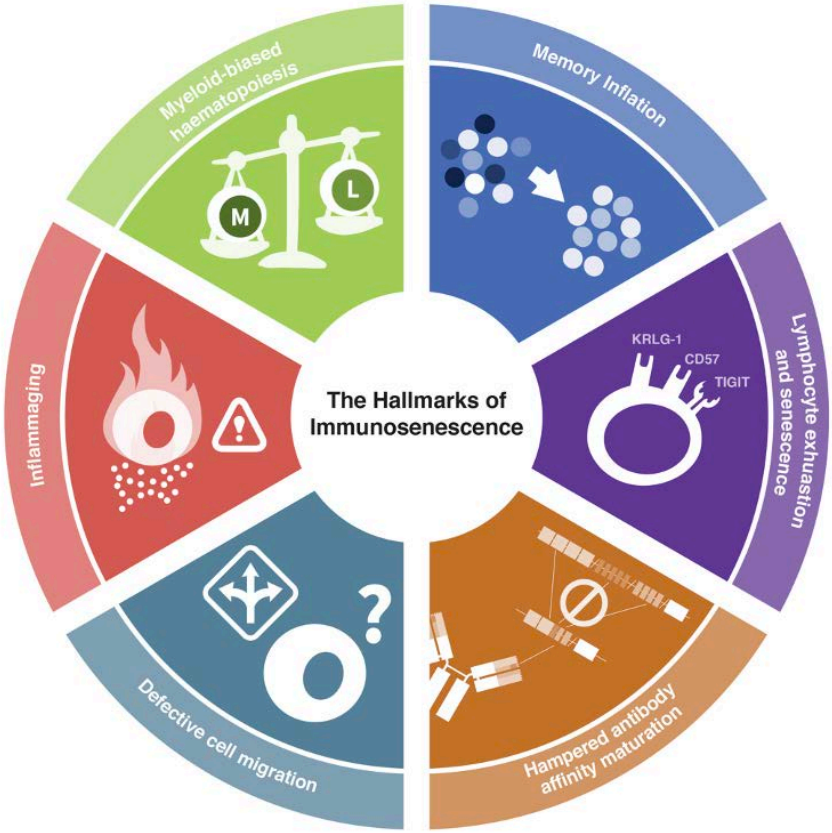

Figure 1. The hallmarks of immunosenescence. *These six phenomena or conditions are the main features that describe the detrimental changes of the immunosenescence process.*

1.1.1 T lymphocytes

One of the most noticeable changes with aging is the accumulation of senescent T cells, characterised by a lost or decreased CD28 and high CD27 expression, short telomers, and lost telomerase activity. As a result, these cells have a reduced response upon stimuli (Bajaj et al., 2021). Increased levels of TNF-α have been demonstrated to play a role in the downregulation of CD28 expression in experienced $CD4^+$ T cells (Cianci et al., 2020), while altered TCR signalling and ERK phosphorylation have been shown to cause blunted lymphocyte activation

(Pinti et al., 2016). Paradoxically, aged $CD4^+$ T_{EM} cells present a reduced cytokine production upon stimuli, while terminally differentiated ones show a higher unspecific cytokine activity that contributes to the overall inflammation seen in the elderly (Cianci et al., 2020). Moreover, decreased trafficking and reduced motility have been also shown for T cells in the lymph node (Pinti et al., 2016).

As previously discussed, there is a significant oligoclonal expansion of the $CD8^+$ cell repertoire associated with ageing, particularly against CMV epitopes (Cianci et al., 2020). The resulting loss of naïve T cell diversity contributes to poorer response to vaccination. Additionally, this phenomenon is not only specific to $CD8^+$ T cells, as it has been shown that a reduced number of naïve $CD4^+$ recent thymic emigrants strongly correlates with a diminished response to yellow fever vaccination in older individuals (Schulz et al., 2015).

1.1.2 B Lymphocytes

Although the total number of B cells seems to remain constant with age (Frasca et al., 2017), the proportion of the different subpopulations undergo significant changes. The most compelling is the decreased number of plasma cells (Bajaj et al., 2021); even if increased circulating low-affinity immunoglobulin has also been described. This phenomenon is caused by a reduced turnover and the accumulation of aged B cells, leading to a loss of repertoire that correlates with poor health (Frasca and Blomberg, 2020). One of the mechanisms behind this loss of repertoire is the downregulation of the E47 transcription factor, which in turn dysregulates the expression of AID, therefore impairing somatic hypermutation. These aged B cells are characterised by a reduced ability to recognize and respond to neo-antigens, a reduced capacity to differentiate into plasma cells, and therefore they secrete less antibodies (Frasca and Blomberg, 2020). Interestingly, despite all these changes in B cells with age that negatively impact the response to vaccination, a study from Ju et al. (2018) found that although the antibodies produced by the elderly upon influenza vaccination are less diverse, they have shown to have a higher breadth of binding to HA epitopes than those produced by young individuals. This suggests that, at least in the case of influenza, the humoral response in the elderly seems to rely more on cross-protective B cells. This finding highlights the need to take into account these differences in the immune response when designing vaccines for the elderly as they may differ from those in younger individuals.

In addition to changes in their inherent function, memory B cells in elderly individuals acquire a senescent-associated secretory phenotype. These population of double negative B cells ($CD19^+CD27^-IgD^-$) significantly expands, and it is characterised by a spontaneous secretion of TNF-α (Frasca and Blomberg, 2020). The pro-inflammatory microenvironment that results from this secretion has been proven to be particularly detrimental for vaccine responsiveness, given that it makes these exhausted B cells refractory to further stimuli (Frasca et al., 2017).

1.1.3 Monocytes

The immune changes in the elderly are not restricted to the adaptive immune system, but also have a significant impact on the different subsets that compose the innate response. In turn, this affects the capacity of elderly individuals to control infections and to respond to immunization. In contrast to the decreased numbers of many lymphocytic populations, the number of some innate immune subsets increases or remains stable due to a myeloid-derived replication bias of the aging haematopoietic stem cells (Goronzy and Weyand, 2013). Interpretation of these changes, however, must be done carefully as to avoid misinterpretation. For instance, Nakaya

et al. affirmed that monocytes were increased in the elderly, and found a negative relation between monocytic expression at day 0 post vaccination and the antibody response to it (2015). This seems to contradict others like Hearps et al. (2012) who argue that the total number of monocytes do no experience a dramatic change with age, but is rather the proportion of the three subpopulations, conventional, non-conventional, and intermediate; which is altered. Functionally, monocytes and macrophages undergo severe modifications. For example, expression of HLA and MHC-II molecules is reduced, affecting their capacity to behave as APCs. Moreover, altered responses to TLR stimulation have been shown to induce higher levels of some cytokines like IL-8 (Pinti et al., 2016), while decreasing levels of others such as IL-6 and TNF-α (Bailey et al., 2019). Also, reduction of phagocytosis, ROS production, and hampered response to IFN-γ have been widely documented (Cianci et al., 2020). The overall dysregulation and the accumulation of immune complexes, hormones, free fatty acids, and lipoproteins, induce a generalized pro-inflammatory activation of the different monocytic populations. This is only aggravated by defects in macro-autophagy, causing an unhealthy accumulation of macrophages in tissue (Bajaj et al., 2021).

1.1.4 Neutrophils

Phagocytosis is also affected in neutrophils, and this is both due to a reduced CD16 expression (Cianci et al., 2020), as well as a defective production of ROS (Sauce et al., 2017). Although these changes greatly affect the uptake of opsonized particles, evidence seems to suggest that ingestion of non-opsonized particles remains unaltered (Pinti et al., 2016). Aged neutrophils are also characterized by their inaccurate migration, causing a spread inflammatory response that reaches further from the site of infection. This defect has been proven to be caused by the constitutive activation of the PI3K signalling pathway, and the dysregulation of CXCR4 and CD62L, which in turn affect neutrophil egress, leading to cell accumulation after immune clearance (Pinti et al., 2016). Dysregulation of CD62L and CD11b also correlates with the levels of IL-6, and the response from aged neutrophils to suboptimal stimuli with this cytokine suggests they are pre-activated by it (Sauce et al., 2017). Finally, reduced ability to produce NETs, and to clear methicillin-resistant *Staphylococcus aureus* infection have been documented in a skin infection model on aged mice (Tseng et al., 2012).

1.1.5 Dendritic cells

There is no consensus in the field about the age-related changes in the abundance and proportion of different dendritic cell (DCs) subsets except for Langerhans cells which in elderly individuals are diminished, and that poorly migrate in response to TNF-α (Pinti et al., 2016). Still, both pDCs and mDCs have been shown to present a reduced antigen presentation activity, and a deficient stimulation to $CD4^+$ and $CD8^+$ T cells (Cianci et al., 2020). Functionally, pDCs in the elderly have been found to produce less cytokines such as IL-12, IL-6, and the three families of interferon molecules (Pinti et al., 2016). Although these defects have been associated with reduced response to the influenza vaccine, Fulop et al. argue that poor responsiveness to immunization depends on the overall innate stimulation rather than on a single subset (2018). Follicular DCs have a reduced expression of the FcγRII receptor which in turn leads to a defective formation of the germinal centres, consequently having a negative impact on the humoral response upon vaccination (Cianci et al., 2020).

1.1.6 NK cells

Populations of NK cells present different changes with aging, being the most dramatic the reduction of the $CD56^{bright}$ subset as a result of the diminished output of new cells from the aged bone marrow (Pinti et al., 2016). In the absence of new cells, a well-preserved memory-like NK cell phenotype, defined by the expression of CD94, NKG2C, and CD57, has been described in elderly individuals. Expansion of this population was first associated to CMV infection, nonetheless, the same phenotype was reported in CMV seronegative individuals, suggesting that other factors of the aging immune system contribute to the development of these NK cells (Bayard et al., 2016). On the other hand, $CD56^{dim}$ NK cells increase with age but they have a deficient production of chemokines and a reduced cytotoxicity against MHC-I negative cells, even if IFN-γ production remains the same as those seen in adults (Cianci et al., 2020).

1.2 Inflammaging

Other important component of the immunosenescence phenomenon is inflammaging, first defined in the year 2000 as a "*a global reduction in the capability to cope with a variety of stressors and a concomitant progressive increase in the proinflammatory status.*" (Franceschi et al., 2000). This chronic low-grade inflammation has been linked with multiple age-associated diseases such as coronary heart disease, rheumatoid arthritis, osteoporosis, Alzheimer's disease, and type-2 diabetes (Frasca and Blomberg, 2016). Interestingly, healthy elderly individuals still show, to a lesser degree, signs of this subclinical inflammation even in the absence of any major health condition (Pawelec et al., 2020). Inflammaging has therefore be suggested to be the biological background that, in addition to genetic and environmental components, favours the development of age-associated diseases (Franceschi et al., 2000). Although there is no consensus on the molecules that ultimately define inflammaging, cytokines such as IL-6, IL-1β, IL-8 and TNFα as well as acute phase proteins such as CRP, are usually used as biomarkers of inflammaging (Pinti et al., 2016). In fact, high levels of these molecules have been consistently found as strong predictors of all-cause mortality (Morrisette-Thomas et al., 2014), and have been demonstrated to be detrimental for the response to vaccination against influenza, yellow fever, and hepatitis B (Bajaj et al., 2021).

Globally, inflammaging is frequently described as the aging of the innate immune system, and it can be broadly defined by two distinct phenomena: a low-level chronic production of pro-inflammatory mediators, and the immune paralysis of specific immune functions upon immune challenge (Fulop et al., 2018). However, limiting inflammaging to an only innate phenomenon puts us at risk of underappreciating its complexity and its relationship with immunosenescence, or even with aging as a whole. In fact, inflammaging has been proposed as one of the main pillars in geroscience research, as it has been shown to be governed by multifactorial elements that are greatly intertwined, and which overlap with other age-related changes of different body systems (Sierra, 2016).

Dissecting the causes of inflammaging is therefore a major challenge. One of the main drivers of this dysregulated immune response is the incomplete resolution of the acute inflammatory phase. A recent study has shown that, despite the onset of acute inflammation being similar in young and old individuals, a reduction in the expression of the TIM-4 receptor on the elderly macrophages prevented appropriate resolution by efferocytosis, and therefore, led to the accumulation of pro-inflammatory signalling at the site of stimulation (De Maeyer and Chambers, 2021). Other important element contributing to inflammaging is the increase in the

number of senescent cells in elderly individuals (Bajaj et al., 2021). These senescent cells are characterised by "*having dysfunctional mitochondria, defective autophagy/mitophagy, endoplasmic reticulum stress, activation of inflammasome by cell debris and misplaced self-molecules, defective ubiquitin/proteasome system, and activation of DNA damage response*" (Fulop et al., 2018). When put together, these alterations to the cell homeostasis prompt to an accumulation of DAMPs which in turn triggers a pro-inflammatory response through activation of different cell subsets (Chambers and Akbar, 2020). Moreover, some of these cells acquire a particular phenotype known as senescent-associated secretory phenotype (SASP), which induces a high production of pro-inflammatory cytokines and miRNAs (Frasca et al., 2017).

1.3 Immunosenescence beyond the immune system

So far, all of the differences of the aging immune system that have been reviewed are intrinsic changes to different immune cell populations. Nevertheless, extrinsic changes can also have an important impact on the quality and quantity of the immune response, as well as on its homeostasis in elderly individuals. The interpretation of extrinsic, however, can be done at two different levels. It can refer to the changes in other body organs or tissues that significantly shape the immune system. For instance, the reduction of the lymph node's total size due to the age-related decrease in the numbers of fibroblastic reticular cells and lymphoid endothelial cells that make up this tissue. These changes result in a decreased capacity to maintain naïve T cells, and the disruption of the overall organization of lymph nodes and germinal centres. Other interesting change is the replacement of bone marrow by adipocytes, an alteration that has been associated with impaired haematopoiesis, as well as with secretion of leptin, adiponectin, and pro-inflammatory cytokines (Bajaj et al., 2021).

When interpreted more broadly, extrinsic factors might also include external agents and/or environmental factors that interact or alter the aging immune system. One of such factors is the gut microbiota, which has been documented to change with age although it is still not clear if this happens as a result of aging, or as a determinant of it (Cianci et al., 2020). The work in this field has experienced a boom in the last decade, and a detailed revision would be out of the scope of this review. Still, it is interesting to mention that studies in germ-free mice have shown that the gut microbiome is essential for specific B cell differentiation upon immunization (Cianci et al., 2020). Likewise, certain bacterial species in gnotobiotic mice have been proven to have either a positive or a negative impact on the immune response to oral and parenteral vaccines. To test these hypotheses in humans, interventional studies have been approached by two different routes, namely probiotics and antibiotics. Results from the former have been highly variable, and their interpretation has therefore resulted challenging. Experiments with antibiotics, in contrast, have proven that dysbiosis of the gut microbiome can impair the antibody response to the influenza vaccine in individuals with no pre-existing memory (de Jong et al., 2020).

Another key factor that can affect the response to vaccination are infections. A large body of evidence has demonstrated that concomitant infections at the time of vaccination have a negative impact on the seroconversion rate, or on the post-immunization antibody titre of affected individuals (Zimmermann and Curtis, 2019). Most of these studies, however, have been done in children as most vaccines are expected to be administered in the first years of life. Nonetheless, CMV infection has been found to be particularly relevant in the elderly due to its contribution to several processes of the aging immune system such as inflammaging and memory inflation. CMV has been proven to increase the age-associated inflammation, particularly by induction of B cell-derived TNF-α, which in turn promotes the transcription of

its viral early promoters (Pinti et al., 2016). Also, CMV is considered one of the main drivers of T cell memory inflation, affecting both αβ and γδ T cells (Pinti et al., 2016). Regarding the effect of CMV infection on vaccination, the results are rather contrasting. Most evidence seems to suggest that CMV seropositivity has a negative impact on vaccine responsiveness both in young and elderly individuals, but particularly for the latter due to the influence of CMV on immunosenescence as a whole. Moreover, studies focusing on aged individuals found that CMV serostatus has a greater impact on the antibody response to vaccination than other inflammatory markers such as IL-6 (Aiello et al., 2017). On the other hand, a higher HAI in a CMV^+ group compared to the control group has been reported, and claimed to be due to the positive effect of infection on keeping a high immune alertness (Fulop et al., 2018).

Not only ongoing infections are able to reshape the immune response. The last decade has seen compelling progress in the study of *trained immunity,* which can be defined as the enhanced responsiveness of the innate immune cells as a result of their previous activation. However, Franceschi et al. (2017) propose that the same components that define trained immunity, namely intensity, type, and temporal sequence of antigenic stimuli; are not exclusive of the innate immune system, but can also apply to the immune system as a whole. In other words, they argue that an individual's response will not only be contingent to its current state, but it will also depend on the nature and intensity of the previous immune challenges it has faced in the past. Given that each individual's immune history is unique, this new concept has been named *immunobiography*. Accordingly, the fact that different immunobiographies can explain some of the variation seen in the immune response within a population, makes it of particular relevance for the elderly, as it can be expected that the singularity of each individual's immunobiography will increase with age. Advancements in this field suggest that the study of the immune response in the elderly should also consider an individual's previous history of infections, an approach that might explain some of the conflicting results that have been discussed so far in this review (Pawelec, 2020).

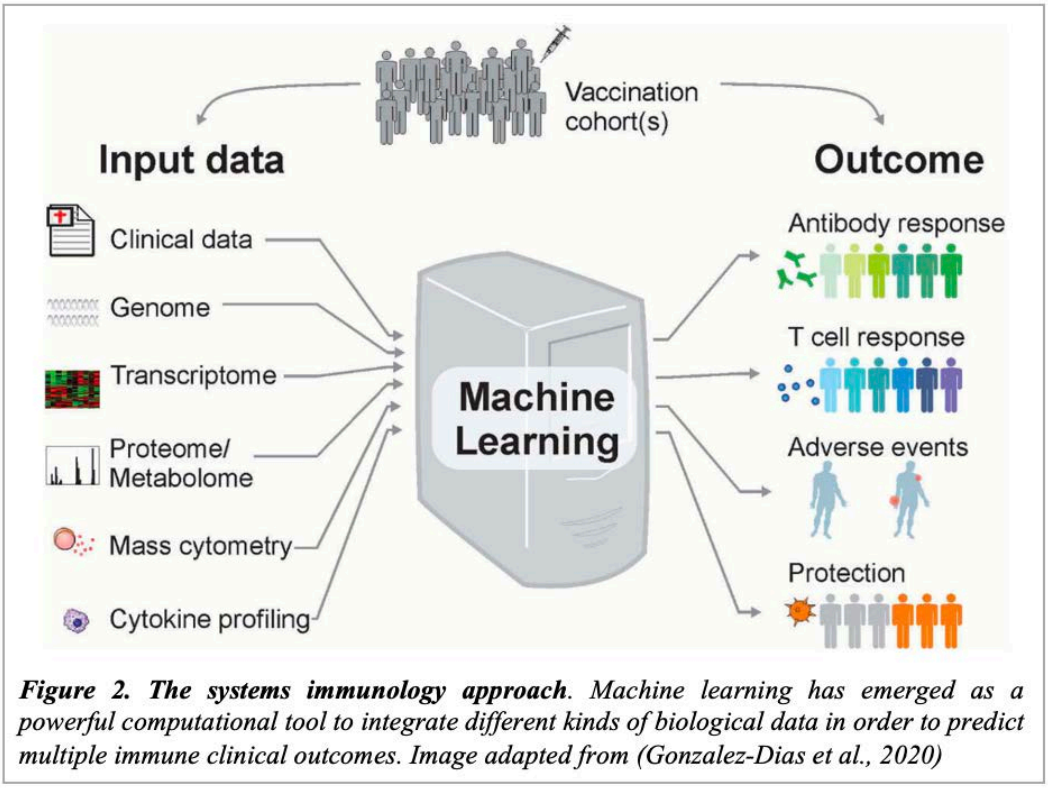

***Figure 2. The systems immunology approach**. Machine learning has emerged as a powerful computational tool to integrate different kinds of biological data in order to predict multiple immune clinical outcomes. Image adapted from (Gonzalez-Dias et al., 2020)*

1.4 Systems immunology: the new horizon

Despite our progress at identifying several age-related changes that impact the elderly immune response, determining which and how exactly these alterations are responsible for the heterogeneous and defective response to vaccination remains to be answered. Interest for this problem has not been an exclusively academic matter; in fact, the need of the pharmaceutical industry to fill in the existent gap between pre-clinical and clinical studies, gave origin to the "translational sciences" in the early 2000's (Ahmed et al., 2012). Development of this field boosted the search for biomarkers that could help understanding and predicting the immune response mechanisms, as well as finding surrogates or correlates of protection that would allow its assessment. These studies resulted in important discoveries such as the correlation between AID expression and the B cell response in elderly against the influenza vaccine; the functional quality decrease of the opsonophagocytic antibodies after Pneumococcus immunization; and the correlation between $CD14^{+}CD16^{++}$ monocytes and the probability of developing systemic adverse effects after Yellow Fever vaccination in individuals 60 years or older (Ahmed et al., 2012).

One of the main obstacles during the early stages of research in this field was to search for new biomarkers using a hit-or-miss strategy. Despite efforts being knowledge-driven, looking for biomarkers with such an approach is like looking for a needle in a haystack; with even greater complexity in the case of the ageing immune system in which, rather than a single factor, changes are explained by the interaction of multiple factors. An answer to this problem came with the development of new transcriptomic and DNA microarray technologies which permitted further exploration beyond what flow cytometry had allowed at the time (Haining and Wherry, 2010). This approach let scientists in the oncology field to develop prognosis predictors based on gene expression signatures, an idea later applied in immunology to explain the heterogeneity of the immune response (Ahmed et al., 2012). Later on, the development of new technologies and the boom of the "omics" supported a larger, more holistic exploration of the dynamics behind the immune response (*Figure 2*). This new method integrating large volumes of data from different components of a given biological system was named *systems immunology*, a term derived from the broader systems biology approached used in other fields of the biomedical sciences.

When applied specifically to vaccines, "the goal of systems vaccinology is to gain a more global representation of the immune response to vaccination, with the hopes of identifying mechanisms of action of current successful vaccines and to use this information for the rational design of novel vaccines" (Sai S. Duraisingham, 2012). In contrast to the hit-or-miss approach mentioned earlier, systems vaccinology is a data-driven method, and therefore, it relies on the measurement of large amounts and varieties of biological data. This complex data requires more robust computational methods than those previously used, which is why the systems biology field would not have been able to advance without the simultaneous development of machine learning algorithms. In addition to the traditional regression analyses, immunologist have begun to explore other algorithms such as support vector machines (SVM), artificial neural networks, Logistic Multiple Network-constrained Regression, and the most recent Sequential Iterative Modelling Over-Night, also known as SIMON (Gonzalez-Dias et al., 2020). This novel approach has led to striking results including the discovery of new biomarkers and gene signatures that can be used as correlates of protection or predictors of clinical outcomes (Li et al., 2017; Nakaya et al., 2011). *Table 1*. summarizes some of the most

relevant results obtained using this approach for different vaccines, privileging those in which elderly individuals were included in the study cohort.

Research in this field still faces some limitations that have prevented researchers from filling the gaps in our understanding of the immune response to vaccination in the elderly. One of them being the sample size of the clinical trials. As seen in *Table 1*, most of the study cohorts count with less than 100 participants, and some of them do not even include elderly people. This represents a challenge in two fronts. First, the low number of elderly patients decrease the probability of finding subtle differences that might allow to discriminate this population from their younger counterparts. Second, having a low number of individuals but a large number of features measured (as in the case of transcriptomics), leads to the phenomenon known as the curse of dimensionality (Gonzalez-Dias et al., 2020), which increases the risk of overfitting the machine learning algorithm resulting in a poor predictive performance. Issues of overfitting can arise as well from the fact that most of these studies are validated through in-cohort cross-validation, and therefore, their predictive power when outside of the particular conditions of the clinical study remain unknown. Another critique to the results in this field is the variability in the timing in which samples are taken. Are the changes reported an immediate variation of the immune response? Or are they stable changes before, during, and after the response? Finally, it will be important for future clinical trials to be designed in a way that a more diverse genetic and social population is included in order to capture a more realistic range of immune variation.

Despite the challenges yet to overcome, the relevance and value of the "systems" approach have become even more evident nowadays with the SARS-CoV-2 pandemic. By the end of 2020, Arunachalam et al. published a study in which PBMC phenotyping and transcriptomic data of healthy versus COVID-19 patients was analysed using this methodology. The authors found that PBMCs from COVID-19 patients presented a decreased expression of HLA-DR, as well as myeloid-derived proinflammatory cytokines. Levels in plasma of EN-RAGE, TNFSF14, and oncostatin M were also found to correlate with disease severity. These results have highlighted possible mechanisms to explain the pathophysiology of the SARS-CoV-2 infection, and have revealed potential targets for a therapeutic intervention. Conceivably, applying the same strategy to a cohort including a robust number of elderly individuals will surely shed some lights on the differences in the immune response among different age groups, and can lead as well to an improved and more rational vaccine design. As different cytometry technologies and next generation sequencing (NGS) platforms become more accessible, it is reasonable to think that application of the systems approach will keep expanding. The future of data-driven vaccine optimization and personalized immune profiling has already begun.

Table 1. Summary of main findings using the system vaccinology approach

Vaccine Model	Study Cohort (n)	Main Findings	Ref.
Influenza (LAIV and TIV)	Young healthy adults (18–50) during the three consecutive influenza seasons (n = 67)	Molecular signatures at baseline were shown to correlate with later levels of antibody titres. These signatures, composed of a network of genes related to inflammatory and antimicrobial responses, were used to develop a DAMIP model with a predictive accuracy of ~90%.	(Nakaya et al., 2011)
Influenza (TIV)	Single cohort of healthy ambulatory subjects ages 20 to >89 years (n = 91)	Use of a machine learning approach to determine a positive correlation between a high antibody response to vaccination, and a particular gene cluster related to lipid biosynthesis. Testosterone, a negative regulator of some of those genes, was shown to have an inverse correlation with antibody titre, unravelling a possible mechanism to explain sex-based immune response differences.	(Furman et al., 2014)
Hepatatis B (HBsAg)	Healthy adults from 25 to 40 (n = 30) or >65 (n = 144)	Development of a transcriptomics-based prediction model for vaccine response based on baseline measurements, with a 65% accuracy rate. Identification of gene signatures of increased B cell responses at baseline that positively correlate with vaccine response. In contrast, inflammatory response transcripts, and frequency of pro-inflammatory innate cells was found to be associated with poorer responses.	(Fourati et al., 2016)
Malaria (RTS,S and rAd35)	Malaria-naïve healthy adults (n = 46). Two immunization regimens: RRR or ARR	Protection conferred by different immunization regimens is associated with regimen-specific correlates of protection. Molecular signatures of B and plasma cells correlated with high antibody response only in subjects vaccinated with the RRR regimen, whereas innate immunity and dendritic cell activation signatures were associated with it only in the ARR regimen. Overall, results suggest that there can be multiple mechanisms leading to protection against *P. falciparum*.	(Kazmin et al., 2017)
Influenza (TIV)	Multi-cohort across five consequtive Influenza seasons. Indivuduals below 35 and above 60 (n = 275)	Identification of nine genes and three gene modules that were significantly associated with the magnitude of the antibody response, and which were validated in an independent cohort. Nonetheless, an inverse correlation was found between the effect of these gene signatures in young and older individuals, meaning that those gene signatures correlated with a better response in the young, are associated with worse responses in older individuals.	(Avey et al., 2017)
Ebola (rVSV-ZEBOV)	Healthy adult subjects (n = 20)	Innate immune signatures based on different cell subsets such as monocytes, dendritic cells, and NK cells; as well as early innate markers, particularly IP-10, were found to correlate with the Ebola vaccine-specific antibody response. It was also shown that IP-10 expression levels on day three after vaccination behaves as an independent correlate of the antibody response.	(Rechtien et al., 2017)
Shingles (Zostavax)	Healthy adults (n = 33) and elderly (n = 44) subjects	Immune and metabolic correlates of vaccine protection were identified. The inositol phosphate, sterol, and glycerophospholipids metabolism networks are strongly related to immune protection. The proposed MMRN model shows a marked association between immune signatures derived from the transcriptomic and metabolomic datasets.	(Li et al., 2017)
Influenza (TIV), Yellow Fever (YF-17D), and SLE	Multi-cohort comparison. Healthy adult subjects (n = 10-96 per cohort)	The peripheral blood signature at baseline that predicts antibody response to the influenza vaccine can also predict the protective response to yellow fever vaccination. Similarly, this same pathway assessed in quiescence correlates with flare episodes in SLE patients.	(Kotliarov et al., 2020)

LAIV: Live Attenuated Influenza Vaccine; TIV: Trivalent Inactivated Influenza Vaccine; DAMIP: Discriminant Analysis via Mixed Integer Programming; rAd35: recombinant Adenovirus 35 ; RRR: three doses of RTS,S immunization regime; ARR: one dose of rAd35 followed by two doses of RTS,S/AS01 immunization regime; MMRN: Multifactorial Response Network .

2. Aim of the study

This thesis takes place in the frame of the Vaccines and InfecTious diseases in the Ageing population (VITAL) project, a European research consortium that aims at providing evidence-based knowledge on vaccination strategies for the elderly population. For achieve this, the consortium has established two main working axes: the study of the mechanisms mediating immunosenescence, and the assessment of the burden of infectious diseases in the elderly population. In line with the former, this study focuses on the effects of inflammaging on vaccine responsiveness, and it aims to develop a prediction model that will anticipate the quality of the immune response to the influenza vaccine. Such model will be based on the baseline levels of 25 inflammation biomarkers measured in serum from participants of the VITAL clinical cohort. Having an accurate prediction model of vaccine responsiveness simplifies the identification of the variables that contribute the most to the predicted outcome, giving us a better insight at those components of inflammation that are most detrimental for the immune response. Furthermore, such model can be as well a powerful public health tool to identify the best target population for vaccination policies.

The VITAL project was initially presented in 2019, and its main study targets were the influenza and pneumococcal vaccines given their relevance for the elderly population. Nonetheless, amidst the global SARS-CoV-2 pandemic, we realized the potential of the VITAL cohort as a framework to study the elderly immune response against the SARS-CoV-2 vaccines, and its differences with the response observed in other age populations. Consequently, the second aim of this study was to develop a spectral cytometry immunophenotyping panel that allows the characterization of the immune response to the SARS-CoV-2 vaccines in human PBMCs. Such panel will allow the identification of the kind of immune response required to confer protection after immunization.

3. Materials and Methods

3.1 Clinical study and sampling protocol

Samples used in this project were obtained as described by the clinical protocol "Immune responses to influenza and pneumococcal conjugate vaccines in older adults compared to middle-aged adults and adults". This study has received approval by the Medical Ethical Committee at the University Medical Centre (UMC) Utrecht (The Netherlands) under registration NL69701.041.19. Elderly participants were recruited by the Spaarne Hospital in Hoofddorp (The Netherlands) from different cohorts of previous studies on Influenza-like illness. Adult and pre-elderly subjects recruited were mainly health care workers and personnel at the UMC Utrecht, and the Utrecht Science Park-Bilthoven, that were eligible for a yearly seasonal influenza vaccine through their employer. The main inclusion criteria were to have been vaccinated against seasonal influenza for the previous season, and to never have been administered a conjugated pneumococcal vaccination. Main exclusion criteria were the use of high-dose or frequent use of corticosteroids; and any medical condition for which procedures in the study protocol could pose a significant risk. Serum samples were obtained by centrifugation after venepuncture 0 to 8 weeks before vaccination with the seasonal quadrivalent inactivated influenza vaccine (QIV) (2019-2020).

3.2 Measurement of inflammation biomarkers

Levels of different inflammatory biomarkers were measured in serum of each study subject obtained as mentioned above. According to their concentration range, cytokines were measured using different methodologies as follows:

- Neopterin was measured using the competitive ELISA kit by Tecan (RE59321). Assay protocol was performed as per manufacturer instructions with exception of the orbital shaker speed which was adjusted to 430 rpm. Concentrations were calculated based on the logistic regression obtained from duplicates of the standard curve.

- Measurement of iFABP2 was done using R&D Systems™ indirect ELISA kit (DFBP20) following the manufacturer's protocol modifying the speed of orbital shaking during incubation to 430 rpm. Concentrations were calculated from extrapolation of the absorbance data on the linear regression equation obtained from duplicates of the standard curve. Sample absorbance for Neopterin and iFAPB2 were measured in an Infinite® 200 PRO plate reader (Tecan) at 450 nm with wavelength correction based on values at 540 nm.

- GM-CSF levels were determined using single molecule arrays (Simoa®) with the GM-CSF 2.0 Reagent Kit (REF 102329) developed by Quanterix™. Protocol was run as per kit's instructions using the Simoa® HD-1 analyzer. Samples were measured in two different runs based on the capacity of the equipment. Concentrations were determined according to the equipment's pre-determined fit equation based on duplicate values of the standard curve.

- Measurements of TNF-α, IFN-γ, IL-1β, IL-10, and IL-6 were performed using a customized Human CorPlex Cytokine Panel kit (97-0329) by Quanterix™ as per manufacturer's instructions besides a reduction in the initial serum centrifugation speed which was adjusted to 4600xg based on equipment availability. Imaging and analysis of these cytokines was done using the Quanterix SP-X™ system. Concentrations were determined according to the equipment's pre-determined fit equation based on triplicate values of the standard curve. The value of the most concentrated point of the standard curve was omitted from the calculations whenever $R^2 < 0.98$.

Levels of C-reactive protein (CRP), CD14, Calprotectin, IL-8, Elastase, Proteinase 3 (PR3), Angiopoietin 2 (Angpt-2), Interleukin 1 receptor agonist (IL-1Ra), CD25, C5a, CCL2, CD163, CXCL10, GP130, IL-6R, Pentraxin-3 (PTX3), and Chitinase-3-like protein 1 (YKL-40); were measured by Luminex or ELISA and were performed by Dr. Yannick van Sleen at the University Medical Center Groningen (Groningen, the Netherlands) in the frame of the VITAL project. Concentration boxplots were created using Prism GraphPad version 7.0a.

3.3 Statistical analysis and prediction models

For the purposes of the statistical analyses and the data interpretation, subjects were grouped in different age categories as follows: Adults (18-49 years old), Pre-Elderly (50-64 years old), and Elderly (<65 years old). Normal distributions for each of the inflammation biomarkers measured was tested with the Shapiro test, followed by Log_{10} transformation of all variables with the exception of age. Analysis of variance was done using the Kruskal-Wallis test with the Holm correction method. Significance was determined as an adjusted *p-value* > 0.05. Post-

hoc pairwise comparison was performed using the Dunn's test, and the Mann-Whitney U test was used for single pairwise comparisons by sex within age groups.
Spearman correlation was calculated on the Log_{10} transformed data. The significance level was corrected by dividing α by the total number of comparisons. Principal component analysis (PCA) was carried out using the *FactoMineR*, and the *factoextra* packages in R. Individuals with no age information were excluded from the analysis *(n* = 311), for the other variables with missing values, the missMDA package was used to impute values that would not affect the loadings' values.

To create a prediction model based on the parameters measured, a Lasso logistic regression and a support vector machine (SVM) algorithms were used. Both models were based on the data from subjects with no missing data (*n* = 227). To run the model simulation, subjects were randomly assigned as good or poor responders based on the proportion of responders and non-responders reported by Furman et al. (2013). Given the lack of data on the response rate of pre-elderly individuals, this age category was excluded from the models. The dataset was divided in training and testing sets at a proportion of 0.8 and 0.2 respectively. Both models were trained using a 5-fold cross-validation. The Lasso regression was programmed using the *caret* package and tuned using the optimized lambda and kappa values. On the other hand, the SVM model was created using the *e1071* package, and using a linear kernel.

All the statistical analyses, and program modelling were performed using RStudio© Version 1.2.5033.

3.4 Spectral cytometry

To optimize an immunophenotyping panel to assess the immune response against the SARS-CoV-2 vaccine, cells from healthy donors were stimulated under different conditions, and later stained and analysed using spectral cytometry. For this, frozen PBMCs were thawed in 10 mL of warm R10+ media (RPMI 10% FBS supplemented with 1% sodium pyruvate, 1% L-glutamine, 1% essential amino acids, and 1% penicillin-streptomycin) centrifuged at 1600 x *g* for 6 min, and later resuspend in 10 mL of R10+. 20 uL of cells were diluted 1:2 in Trypan blue and counted on a Kova counting chamber (87144, Kova International). The cells were then centrifuged and resuspended in order to dispatch $2x10^6$ cells in 500 uL of R10+ in sterile 5mL round bottom polystyrene FACS tubes. 10 uL of anti-CD107a (Table X) was added to each of the tubes, which were then treated under a different stimulation condition, namely Staphylococcus enterotoxin B (SEB) 6 ug/mL (s4881, Sigma-Aldrich), 2 uL of Dynabeads™ Mouse T-Activator CD3/CD28 (11452D, ThermoFisher Scientific), Concanavalin A 5 ug/mL (C5275, Sigma-Aldrich), or R10+ as a negative control. The cells were incubated for 1 hour at 37 ºC followed by addition of Brefeldin A (B6542, Sigma-Aldrich), and Monensin (m5273, Sigma-Aldrich) for a final concentration of 5 ug/mL each. Incubation at 37 ºC was continued overnight. 12-14 h post stimulation, cells were rinsed and the *Pheno Mix* antibody mix (*Table 2.*) was added and left for incubation for 15 min in the dark. Afterward cells were washed with a 2% FBS solution in PBS, and permeabilize using 200 uL of Cytofix/Cytoperm (554714, BD Biosciences) for 20 min. Next cells were washed twice using the Perm/Wash solution (554714, BD Biosciences) diluted 1:10 as per manufacturer's instructions. Cells were then incubated at 4ºC for 20 min with the *Function Mix* (*Table 2.*), rinsed two times with the Perm Wash solution mentioned above, and fixed with a 1% PFA solution.

FACS was performed on an Cytek™ Aurora spectral cytometer (Cytek Biosciences). Compensation matrix for spectral unmixing was done using Anti-Mouse Ig, κ/Negative

compensation beads stained with the antibodies on *Table 2*. Data was initially processed and unmixed using the SpectroFlo® Software (Cytek Biosciences) and exported as a .fcs file for further analysis on FlowJo v.10.7.2 (Beckton, Dickinson & Company).

***Table 2**. Fluorochrome-conjugated antibodies for spectral cytometry.*

	Antigenic Target	Conjugated Fluorochrome	Volume (uL)	Manufacturer	Reference
	CD107A	VioBlue	10	Miltenyi	130095520
Pheno Mix	CD4	BUV395	5	BD Horizon	563550
	CD45RA	BUV563	1.5	BD Biosciences	612926
	CD8	AF405	3	Invitrogen	MHCD0826
	CD3	Pac. Orange	3	Invitrogen	CD0330
	CD28	BV605	5	BD Biosciences	562976
	PD1	BV711	5	BioLegend	329928
	CD57	PeCy5	5	Abcam	ab25445
	CD27	APC-Cy7	2	BioLegend	302816
Function Mix	IL-8	BV510	5	BD Horizon	563311
	IL-2	BV785	1.5	BioLegend	500347
	MIP1b	FITC	12	R&D Systems	IC271F
	TGFb	PerCPCy5.5	5	BD Pharmingen	562423
	IL-1b	PE	3	Invitrogen	12-7018-82
	Perforin	PE-Dzz594	1.5	BioLegend	308131
	TNFa	PeCy7	3	BD Pharmingen	557647
	IL-4	APC	1.5	BioLegend	500811
	GzmB	AF647	3	BD Pharmingen	560212
	IFNg	AF700	2	BD Pharmingen	557995

***Phenomix** refers to the antibody mix staining surface markers.*
***Function mix** refers to the antibody mix used after cell permeabilization to stain production of different immune mediators.*

4. Results

4.1 VITAL cohort characteristics

To characterize how the inflammatory status pre-vaccination might affect an individual's response to immunization, a panel of 25 inflammatory-associated biomarkers were measured in 316 ambulatory healthy subjects that took part of the VITAL clinical trial. Almost half of the participants were elderly individuals, and the male to female ratio varied in each of the three age groups. On average, participants' body mass index (BMI) was normal for the adult and pre-elderly groups, while for the elderly the average was slightly above the normal range (*Table 3.*).

***Table 3**. VITAL cohort characteristics.*

	Adult (<50 years)	Pre-Elderly (50-65 years)	Elderly (>65)
n	58	95	158
Age	35.8 (±7.6)	57.9 (±4.0)	75.6 (±7.1)
Male:Female	0.53	0.70	1.14
BMI	24.0 (±3.5)	24.6 (±3.6)	26.0 (±4.2)

4.2 The specific increase of different inflammatory biomarkers with age is described by three distinct trajectories.

Consistent with the development of inflammaging with age, the concentration of 18 out of the 25 measured biomarkers was significantly higher in the elderly as compared to the adult group (*Supplementary Table 1*). However, when considering the levels of inflammation in the pre-elderly, it is evident that the time (i.e., the age) at which each biomarker starts progressing towards a more inflammatory state is not the same for all the measured molecules. According to the level of inflammation in the pre-elderly, the increase of each biomarker with age can be described by one of three different trajectories. First are those for which the concentration progressively increased with age, with pre-elderly presenting a distinct intermediate level of inflammation between the adult and the elderly (*Figure 3A*). IL-6, neopterin, YKL-40, CXCL10, and CD163 belonged to this category. Second are those for which the pre-elderly average concentration was significantly higher than the one measured in adults but indistinguishable from that of the elderly (*Figure 3B*). This category includes IL-8, CCL2, GP130, IL-6R, and iFABP2. Finally, a third category is defined by those biomarkers for which no significant differences were observed between adult and pre-elderly despite the high inflammation levels in the elderly (*Figure 3C*). This was the case for CRP, CD14, PR3, Angpt-2, CD25, IL-1RA, and Elastase.

In contrast to the molecules in these three categories, no significant differences in the concentrations of GM-CSF, IL-1β, TNFα, and IL-10 was detected across age groups (*Figure 3D*). These results prove that the increase in the age-associated inflammation behaves differently for each of the biomarkers here studied, and suggest that the onset of inflammaging might begin before and individual reaches an old age. Interestingly, there was no significant variation in the concentration of these biomarkers when comparing male and female subjects within each age group (*data not shown)*, with the exception of IL-1β in pre-elderly (*p-value=0.008)* for which male participants had higher levels compared to their female counterparts.

4.3 Inflammation positively correlates with age but is not enough to discriminate between age groups.

Association between the biomarkers measured was calculated using Spearman's correlation coefficient, adjusting the *p-value* as described in the *Materials and Methods* section. Out of the 26 parameters included, IL-1RA was the marker that correlated with the largest number of parameters (17), followed by IL-6 (15), age (14), as finally Neopterin and YKL-40 (12 each). The strongest correlation was between elastase and PR3 (0.7). On the contrary, others like GM-CSF and CCL2 did not correlate with any other parameter. Interestingly, most of the correlations were very modest, suggesting that the selection of biomarkers in this study was not redundant, and that it covers different processes of the inflammatory response.

To gain further insight on how these parameters explained the variability between the subjects, the data was analysed using Principal Component Analysis (PCA). The eigenvalues obtained for the first and second principal components shows that IL-1RA, Age, Neopterin, IL-6, and YKL-40 are the parameters that contribute the most to the first dimension as well as to the

overall distribution of the data (*Figure 5. and Supplementary Figure 2A*). On the other hand, Calprotectin, Angpt-2, IL-1β and elastase explain the greatest amount of variance on the second dimension (*Supplementary Figure 2B*). Remarkably, gender was the parameter that contributed the least to explain the observed variance in the first two principal components. Nonetheless, it is important to remember that the two components shown in the graph only explain 25% of the total variation and, therefore, the influence of gender in the total variability should not be discarded.

Finally, subjects on the PCA plot were labelled according to their age to test if it was possible to discriminate between different age groups based on the levels of the different inflammatory markers. Unfortunately, there is significant overlap between the cluster from each of the age groups indicating that these parameters are not enough to accurately group an individual according to his or her age (*Figure 6.*). Despite that, when taken together, *Figure 5*. and *Figure 6*. suggest that our data is consistent with the previous literature in that the cluster with the more proinflammatory profile was the that of the elderly.

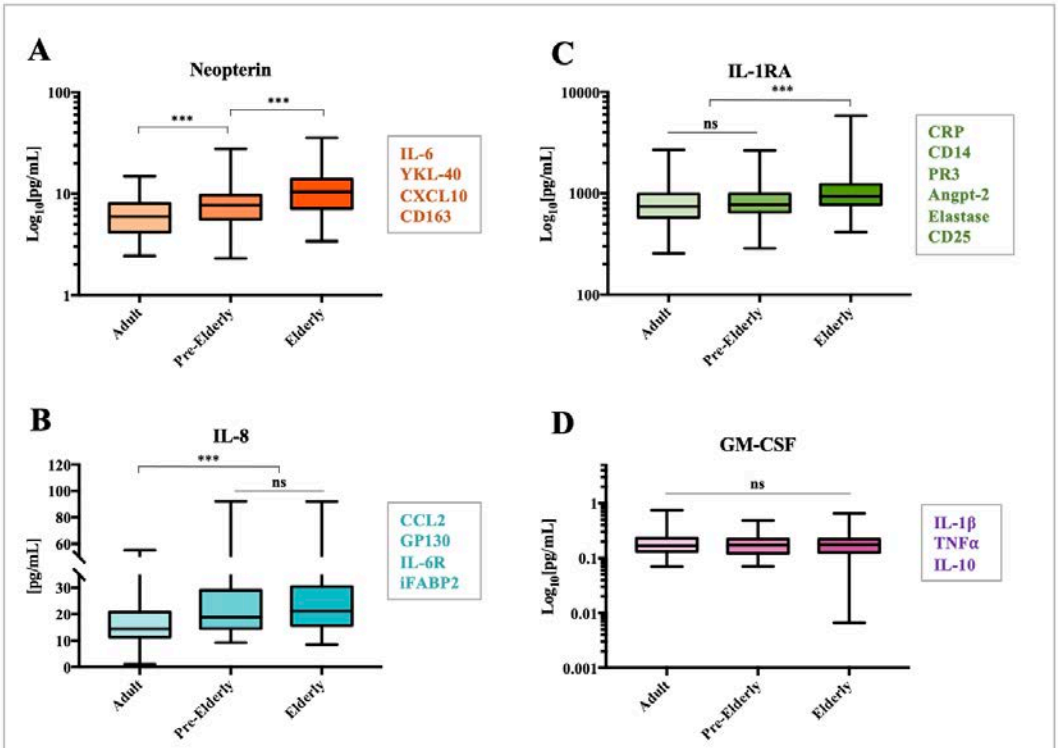

Figure 3. Concentration changes of inflammation-associated biomarkers follow different trajectories across age groups. Concentration by age group of *(A) Neopterin (B) IL-8 (C) IL-1RA (D) GM-CSF. Molecules next to each boxplot indicate other biomarkers that follow a similar distribution by age group. n.s = no significant difference. *** = p-value < 0.001*

4.4 SVM outperforms Lasso logistic regression at predicting vaccine responsiveness

By the time the present work was submitted, information about the subjects' immune response to vaccination was not yet available. Therefore, to be able to develop a predictive model using machine learning, I simulated the data using the rates of vaccine responsiveness reported by Brodin et al. (2015) in a study cohort in which only 38% of the elderly population reached a positive immune response, compared to 80% of adults. These rates were used as the probability that each of the VITAL subjects in my data set had to be randomly assigned as a good responder depending on their age group. Given that there was no specific information for the pre-elderly group, I decided to omit this age category to avoid introducing any unknown bias.

To increase the chances of creating an accurate prediction model, two different machine learning algorithms were used. Lasso logistic regression and support vector machine (SVM) were chosen over others given the nature of my data, as well as these models' simplicity and interpretability. In both cases 80% of the data was used to train the model using a 5-fold cross-validation. When tested using the remaining 20% of the data, a better predictive performance is observed with the SVM model with an accuracy of 0.73 in its predictions, compared to 0.67 prediction accuracy for the lasso logistic regression (*Figure 7*). This difference was mostly due to the higher rate of type I errors in the lasso regression. It is reasonable to think that the accuracy rates of both models will increase once the real data becomes available, however, these results demonstrate that it is possible to develop a prediction tool for vaccine responsiveness based on inflammatory biomarkers.

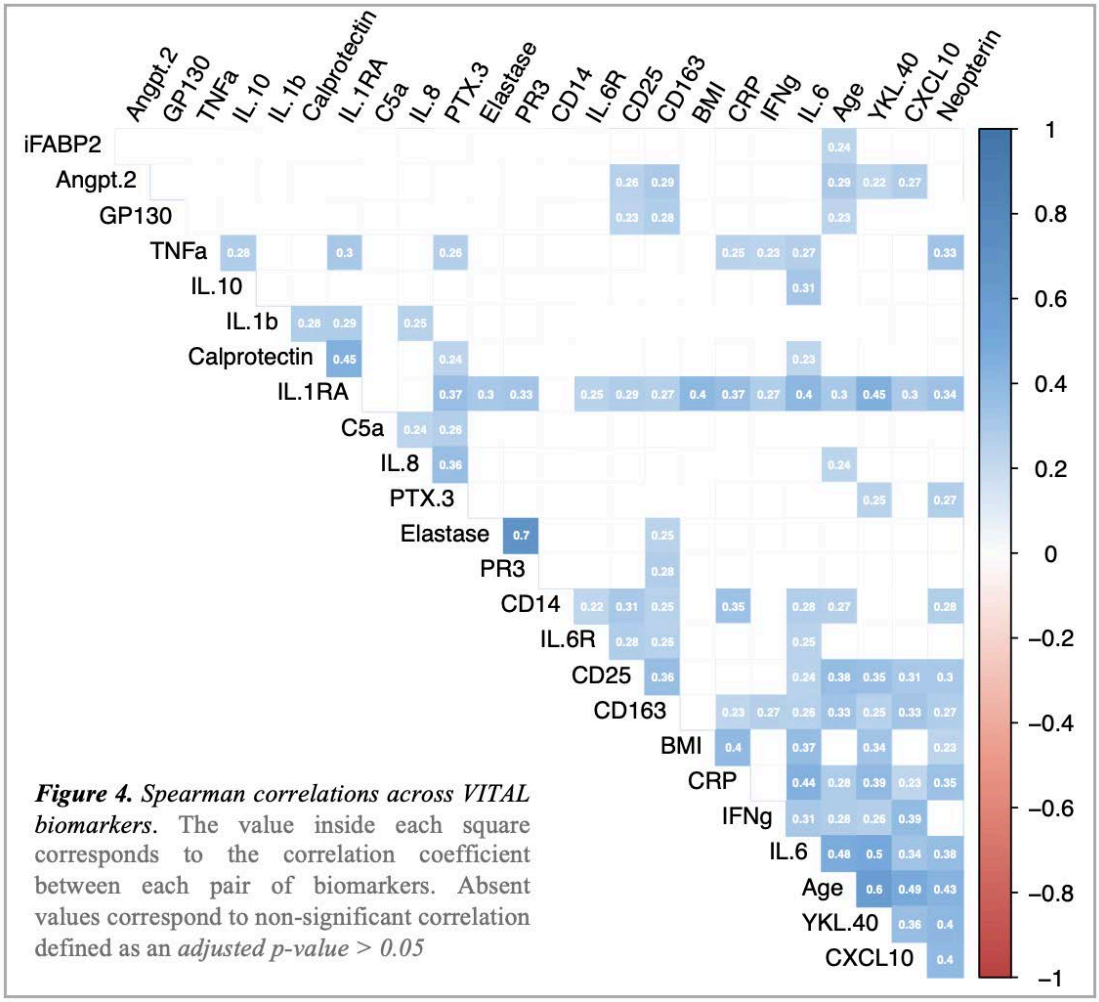

Figure 4. *Spearman correlations across VITAL biomarkers*. The value inside each square corresponds to the correlation coefficient between each pair of biomarkers. Absent values correspond to non-significant correlation defined as an *adjusted p-value > 0.05*

4.5 Spectral cytometry as a potential tool to assess vaccine responsiveness to the SARS-CoV-2 vaccine.

Contrary to the influenza vaccine for which the HAI titre fold-change has been longed been used as a gold standard measure of response to immunization; the correlates and surrogates of protection for the SARS-CoV-2 vaccines remain loosely defined. As a first approach to characterize the immune response to such vaccines, I designed a comprehensive immunophenotyping spectral cytometry panel with 20 markers to try to capture a wider arrange

of immune parameters. (*Table 2*.). To optimize this tool, the panel was tested using human PBMCs stimulated under different conditions, namely concanavalin A (ConA), *Streptococcus* endotoxin B (SEB), and CD3/CD28 magnetic beads as described in the *Materials and Methods* section. Phenotypic analysis of the different T cell subpopulations was done following the gating strategy described on *Supplementary Figure 2*. Noteworthy, a significant increase of the $CD8^+$ T cells was observed under stimulation with ConA, mostly due to an unexpected 2-fold increase in the number of naïve lymphocytes when compared to the unstimulated cells. However, the percentage of T_{CM} and T_{EM} was half of that of the unstimulated group (*Figure 8B*). The proportions of these $CD8^+$ subsets for the SEB or beads treated cells was considerably similar to that of the control group (*Table 5*.). In contrast, total numbers of $CD4^+$ cells in the ConA-treated condition were lower than those in the control, despite the sharp increase in the proportion of naïve lymphocytes in the latter (*Figure 8A*).

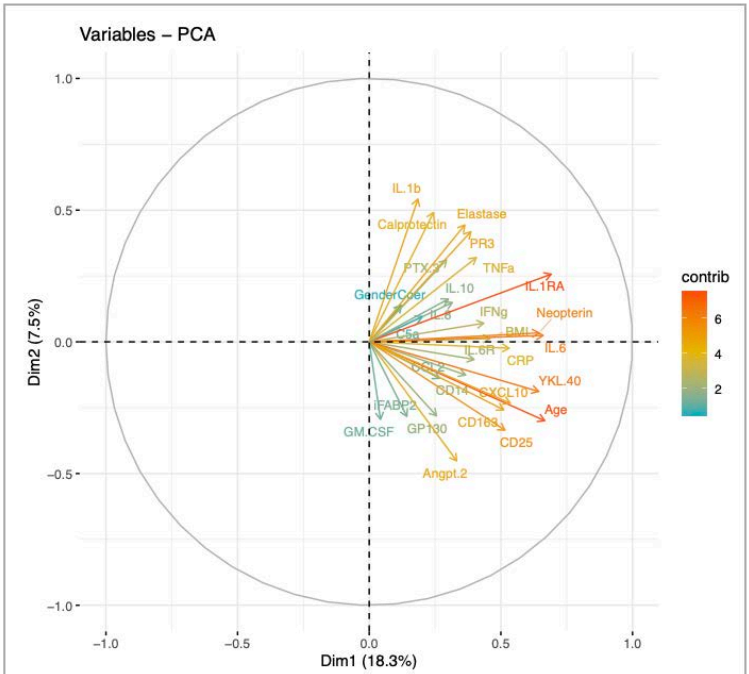

Figure 5. PCA Biplot. Two first principal components can explain around 25% of the total variability in the VITAL cohort. Each vector represents the loading value of each variable in the first and second principal components.

To assess the lymphocyte function upon stimulation, the gating strategy presented in the *Supplementary Figure 3* was used. By evaluating the cytokine secretion profile in each of the conditions (*Table 5*.) it is immediately evident that ConA is consistently the greatest inducer of the immune response under my experimental conditions. Notorious induction of TNFα is seen both in $CD8^+$ and $CD4^+$ cells (*Figure 9A*), as well as robust expression of IL-2 in $CD4^+$ (*Figure 9B*), and MIP1-β mostly in the $CD8^+$. Only very low levels of IL-8 and IL-1β were detected across the different conditions, suggesting that optimization of my staining protocol might be required. Unexpectedly, secretion of granzyme B and perforin seemed to be higher in the unstimulated cells than in any other group, Finally, no CD107a was detected in any of the treatments. Overall, it can be concluded that ConA is the best positive control to assess cytokine induction; nevertheless, it is still necessary to optimize the staining protocol to be able to capture those cytokines that were not properly detected. This experiment confirms the usefulness of spectral cytometry as an approach to evaluate a broad range of phenotypic and functional markers that will be necessary when studying the response to the SARS-CoV-2 vaccines.

5. Discussion:

The application of machine learning methods in the field of immunology has considerably increased in the last decade. This approach has been proven to be a useful tool to explore the mechanisms regulating the immune response, as well as to develop prediction models that support clinical decision-making and public health policy (Furman et al., 2013). This approach has also emerged as a strategy to study the mechanisms behind the ageing of the immune system, a phenomenon for which several factors are known to contribute, but for which a practical model able to discriminate between good and poor responses to vaccination is still lacking. In the present study I developed two inflammation-driven machine learning models with the potential to predict vaccine responsiveness. The construction of such models was based on the levels of 25 inflammation-related biomarkers measured in sera from 316 participants of the VITAL cohort which included subjects from 25 to 92 years old, grouped in three different age categories, namely adult, elderly, and pre-elderly. Inclusion of the latter category separates the VITAL cohort from most clinical studies in which only the age extremes are compared. Considering this intermediate age group provides a valuable insight in the development of inflammaging and its impact at different stages of life. For instance, by examining the concentration increase of the different biomarkers between age groups, I was able to determine that the progression of each biomarker towards a more pro-inflammatory state follows one of three specific types of trajectory (*Figure 3*). These observations suggest that the shift towards inflammaging is gradual, and that it begins before most individuals reach an old age. These findings are notably important for the design of vaccination policies, favouring an earlier immunization schedule for the adult population.

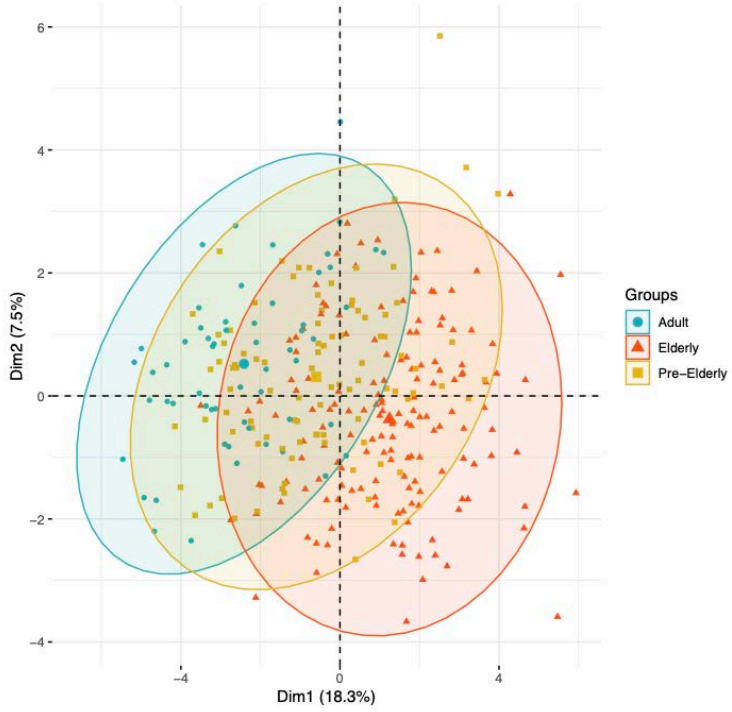

Figure 6. *PCA clustering by age group. Significant overalap of age clusters prevents accurate discrimination of indivuals but their disposition suggest a tendency of elderly individuals towars inflammation*

For those markers which remained stable across the three age groups (*Figure 3D*), it is reasonable to think that differences in the secretion of these molecules might not be evident in the absence of immune stimuli. Moreover, the results in this study are consistent with what has been described by Di Iorio et al. (2003), who showed no association between IL-1β levels at baseline and age, sex, or serum levels of other inflammatory molecules such as IL-6, IL-1Ra, and TNFα. Nonetheless, the same study reported a positive correlation between IL-1β levels and some age-associated diseases such as angina or congestive heart failure. It is therefore important to mention that frailty of the VITAL participants was not included in this study, limiting the possibility to assess the role of these inflammatory biomarkers in healthy ageing compared to pathological ageing (Goronzy and Weyand, 2013).

Figure 7. *Classification performance of each of the prediction models.*

Support Vector Machine

Simulated \ Predicted	NR	R
NR	13	4
R	4	9

Accuracy: 0.73

Lasso Logistic Regression

Simulated \ Predicted	NR	R
NR	14	7
R	3	6

Accuracy: 0.67

R *= Good responder.* ***NR*** *= Poor (non) responder*

A significant positive correlation is however observed between several of the measured biomarkers (*Figure 4*). Not surprisingly, elastase and PR3 had the highest correlation as both serine proteases have been shown to have overlapping substrate specificity, and are both implicated in the degranulation response in neutrophils (Kessenbrock et al., 2008). The overall degree of association of most biomarkers with age was remarkably higher than those previously reported by Morrisette-Thomas et al. (2014), particularly for IL-RA (0.30 vs 0.07), IL-6 (0.48 vs 0.31), and CRP (0.28 vs 0.15). Other markers not included in that study but here measured had even stronger correlations with age, including neopterin (0.43), CXCL10 (0.49), and YKL-40 (0.60); suggesting that our biomarker panel is able to successfully capture the age-related inflammation to a certain extent. These six markers were also identified to be the main discriminant factors of the variability observed in the PCA. Not surprisingly, IL-6 belongs to this group, consistent with the large body of evidence that implicates IL-6 in age-related immune dysregulation and poor vaccine responsiveness (Frasca and Blomberg, 2016). Contributions by neopterin and YKL-40 are an interesting result given that both have been successfully used as predictive markers of long term outcomes for inflammatory-related events or conditions in the elderly (Larsen et al., 2017; Rathcke et al., 2010). The greatest contributor was IL-Ra, consistent with previous studies in which this protein was part of a molecular signature explaining 68% of the cytokine vaccine response variability (Huttner et al., 2019). Moreover, it has been shown in mice that levels of IL-Ra seem to be higher in aged mice, and that this increase relates to a greater susceptibility to infection after vaccination (McDonald et al., 2017). Although the PCA clustering by age (*Figure 6*) does not allow for proper discrimination between different age groups, the fact that IL-Ra, an anti-inflammatory molecule, contributes in the same direction as the other three above-mentioned pro-inflammatory markers, suggests that the variability observed in the data is not just a measure of total inflammation but rather of the overall immune activation.

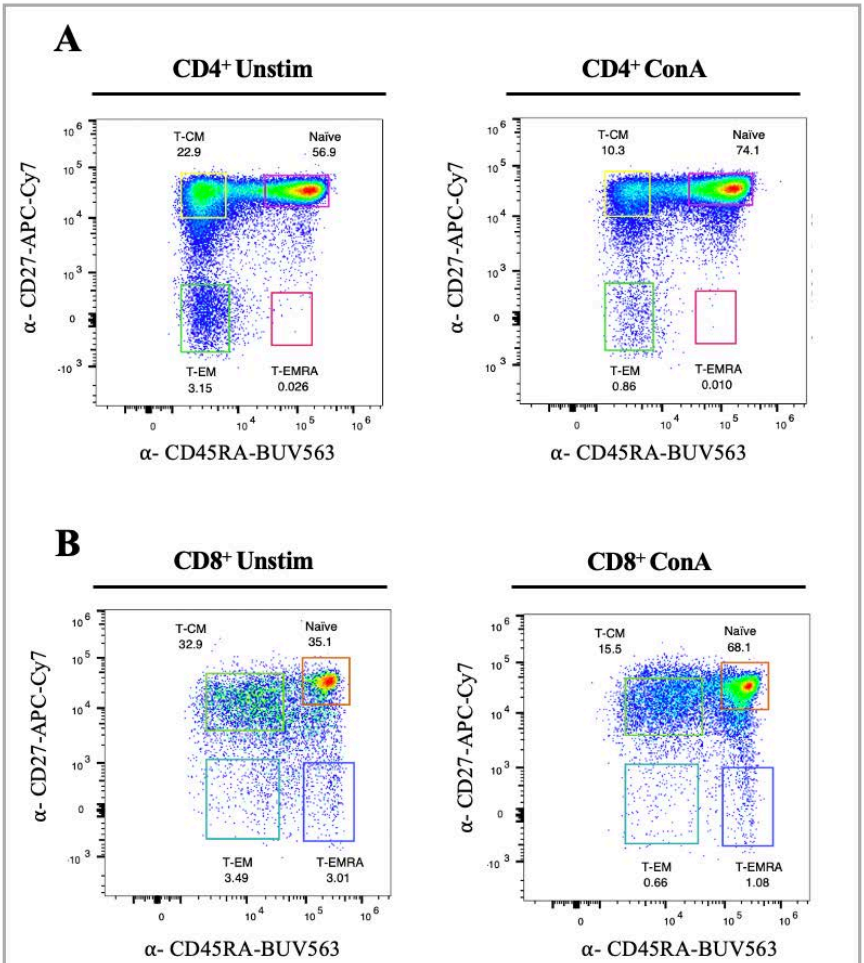

Figure 8. Differences in the proportion of lymphocytic subsets between ConA stimulated cells and unstimulated control . Gating for: (A) $CD4^+$ T cells (B) $CD8^+$ T cells.
T_{CM} = T central memory; T_{EM} = T effector memory; T_{EMRA} = T terminally differentiated effector.

Given that the different machine learning algorithms do not perform equally when given the same data set, two predictive models were developed using different strategies. The first model was a logistic regression, an algorithm frequently used in the analysis of biomedical data (Gonzalez-Dias et al., 2020). The lasso penalty introduced in my model aimed at reducing the set of predictors used, and therefore increasing interpretability. However, this might have given some variables like age, a disproportionate weight on the calculation of the outcome, resulting in a modest 0.67 accuracy rate. The second model was developed using a SVM approach which relies more on the spatial geometry of the data rather than on its statistical probabilities as in the regression. Through this approach I was able to train a model with a 0.73 accuracy rate. Although my results seem to suggest that SMV outperforms the lasso logistic regression, it is important to remember that the data defining good and bad responders was randomly simulated given that the actual data was not available at the time. Significant changes in the accuracy obtained with both models were seen when altering how vaccine responsiveness was assigned (*data not shown*). This indicates that both models are highly sensitive to variation to the outcome variable, and that they must be validated with the real data. Nonetheless, these observations also suggest that improvement in the models' accuracy can be expected once validated.

Overall, this proof-of-concept study demonstrates that it is possible to develop an inflammation-based model to predict vaccine responsiveness to the influenza vaccine in the elderly. To my knowledge, the selection of markers in this study has been the most comprehensive panel of exclusively circulating inflammatory biomarkers that has been used to program such a model, contrasting with previous studies mainly based on transcriptomic analysis (Kazmin et al., 2017; Kotliarov et al., 2020; Morrisette-Thomas et al., 2014; Nakaya et al., 2011). Having a limited set of parameters increases the interpretability of the prediction models and allows the assessment of their particular contribution to the hyporesponsive phenotype. Rather than a personalised diagnostic tool, these prediction models should be able to define an average age range at which the age-related changes to the immune system might put at risk the quality of the response to vaccination. This will certainly have an impact in how vaccination strategies are designed, possibly pointing out at the pre-elderly as a more reasonable target of immunization health policies.

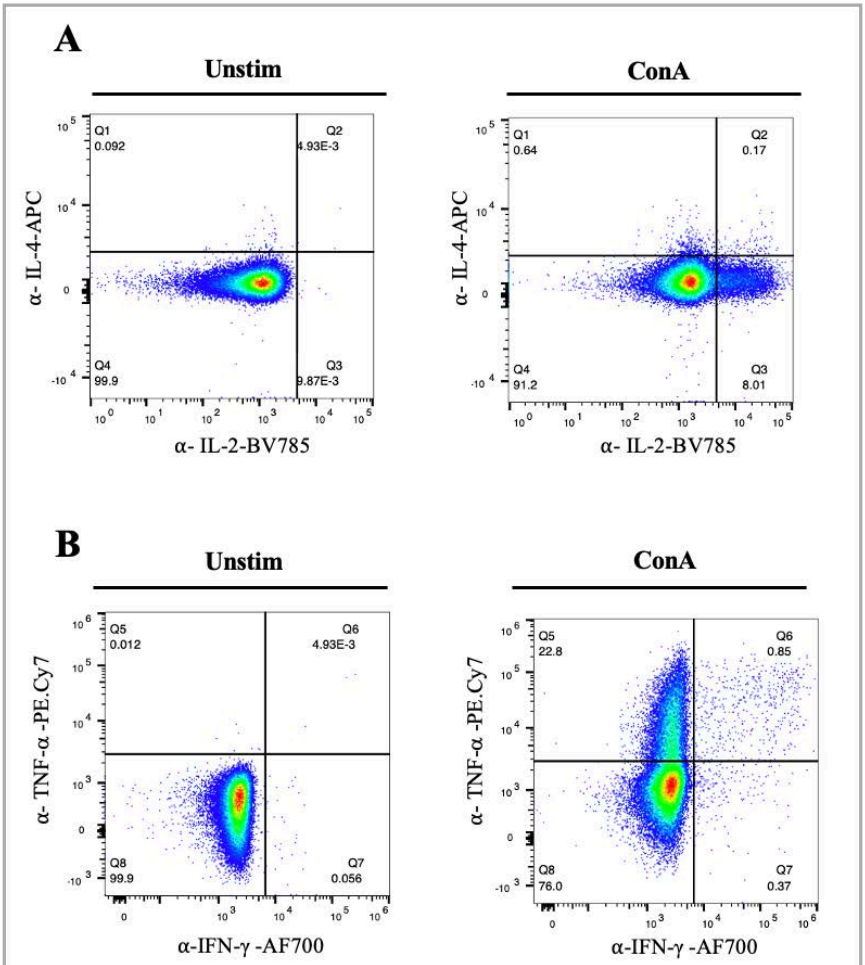

Figure 9. ConA stimulates the production of inflammatory cytokines in $CD4^+$ T cells . Gating for: (A) IL-2 and IL-4 (B) IFN-γ and TNF-α .

The current SARS-CoV-2 pandemic has risen awareness about the need to better understand the differences between the adult and the elderly immune responses. The VITAL cohort is therefore a compelling opportunity to study the relation between inflammation, the ageing immune system, and the response against this virus and its vaccine. The SARS-CoV-2 infection is characterized by a highly inflammatory response, with non-survivors displaying a state termed "stuck in innate immunity" (Bajaj et al., 2021), which has been shown to be more

prevalent in the elderly, and which is defined by excessive production of IFN-α and -γ, CCL2, CXCL10, and other IFN-stimulated genes. Given the role of inflammation in the immune response against this virus, I speculate that a SARS-CoV-2-specific prediction model could be developed following the same approach used here for the influenza vaccine. However, training the algorithm requires the definition of good and poor responders, which in the case of SARS-CoV-2 remains poorly defined. The spectral cytometry panel designed in this study was shown to be an excellent tool to measure multiple phenotypic and function characteristics of human PBMCs from a single sample, a significant improvement from traditional flow cytometry in which the number of parameters that can be simultaneously stained is more limited. I also showed that ConA is an appropriate stimulant to be used as a positive control for production of T cell-derived cytokines. This cytometry panel should be compatible as well with the protocol recently presented by Konstantin Föhse et al. (2021) in which the authors show that T cell-derived IFN-γ, TNF-α, and IL-1β production is decreased in cells from vaccinated subjects as compared to those from convalescent patients.

Table 4. *Phenotypic and functional characterization of PBMCs under different stimulation conditions.*

	CD8+ (%)					CD4+ (%)			
	Unstim	ConA	SEB	Beads		Unstim	ConA	SEB	Beads
Total	10.1	19.6	11.3	10.9	**Total**	62.9	49.3	54.1	60.4
Naïve	35.1	68.1	33.8	36.6	Naïve	56.9	74.1	60.1	56.3
T-CM	32.9	15.5	32.5	32.0	T-CM	22.9	10.3	20.8	23.1
T-EM	3.5	0.7	3.4	3.4	T-EM	3.2	0.9	2.3	2.9
T-EMRA	3.0	1.1	2.8	3.1	T-EMRA	0.0	0.0	0.0	0.0
TNFα		3.0	1.0	2.3	TNFα		22.8	6.1	8.9
IFNγ		1.4	0.5	0.3	IFNγ		0.3	0.1	0.1
TNFα+ IFNγ+		1.9	1.0	0.5	TNFα+ IFNγ+		0.9	0.5	0.5
MIP1β		3.4	2.6	*	MIP1β		0.0	0.0	*
IL-8		0.0	0.0	0.0	IL-8		0.4	0.1	0.0
IL-1β		0.1	0.1	0.2	IL-1β		0.0	0.0	0.2
TGFb		0.1	0.0	*	TGFb		0.0	0.0	*
IL-2		1.1	0.5	0.5	IL-2		8.0	2.6	4.8
IL-4		-0.1	1.2	2.0	IL-4		0.5	0.2	0.1
IL-2+ IL-4+		0.0	0.0	0.0	IL-2+ IL-4+		0.2	0.1	0.2
GzmB		-4.0	2.1	-1.4					
Perforin		-9.5	-0.5	0.4					
Perf+ Gzmb+		-15.5	0.6	0.4					

**Estimation not possible due to interference of the stimulation beads in the gating plot.*

6. Future perspectives:

The next step towards the improvement of the prediction models developed in this study will be to validate or to re-train them using the results from the HAI antibody titre fold change from the VITAL cohort participants instead of the simulated data. This will significantly reduce any potential bias that might have been introduced in the models during the simulations. As with any classification model based on machine learning algorithms, it is possible that optimization of some parameters could have led to model overfitting. Therefore, it will be particularly important to validate the models here developed with other clinical cohorts for which the same parameters were measured.

Improvement of the models' accuracy can be further achieved by including other immune parameters known to affect the response to vaccination in addition to the inflammatory biomarkers here used. For instance, it would be interesting to introduce other variables such as the naïve/ effector memory ratios for $CD8^+$ and $CD4^+$ T cells (Pinti et al., 2016), the abundance of circulating $CD19^+CD27^-IgD^-$ B cells (Frasca et al., 2017), the subjects' CMV serostatus (Aiello et al., 2019), or the gut microbiome diversity (Cianci et al., 2020). It is foreseen that some of this data will become available from other research groups that are part of the VITAL consortium. Inclusion of these criteria will not only increase the model's accuracy, but it will help understand how inflammaging is connected to other immune-related changes in the elderly. Likewise, assessment and inclusion of the subjects' frailty will give us the opportunity to dissect some of the differences between healthy and pathological ageing, and how these differences affect the response to immunization.

If validation with the real data shows that it is indeed possible to accurately predict vaccine responsiveness based on baseline parameters, it would be reasonable to think that by modifying the baseline inflammation we will be able to reshape the response to immunization (Tsang et al., 2020). The prediction models developed in this study have therefore the potential to be used as tools to measure how different immunomodulatory therapeutics can alter the immune homeostasis prior to vaccination, and how these changes will later impact the quality of the response. This combined therapy is a very promising approach, particularly in the elderly, as it stands as an alternative to overcome the hyporesponsiveness in this age group.

Finally, the response to immunisation is vaccine-specific and, therefore, it is expected that the levels of baseline inflammation will have different effects on such response. With the spectral cytometry panel designed in this study it will be possible to characterize how different effector and memory lymphocytes react to the SARS-CoV-2 peptides after infection or vaccination. To assess this experimentally it will be necessary to incubate human PBMC with multiple peptides spanning across the sequence of the viral Spike protein. Using the spectral cytometry panel will make evident how the immune response to the virus differs between naïve, convalescent, and immunized subjects. Similarly, it will be possible to assess the differences between young and elderly individuals, and to determine the impact that baseline inflammation has on the immune response.

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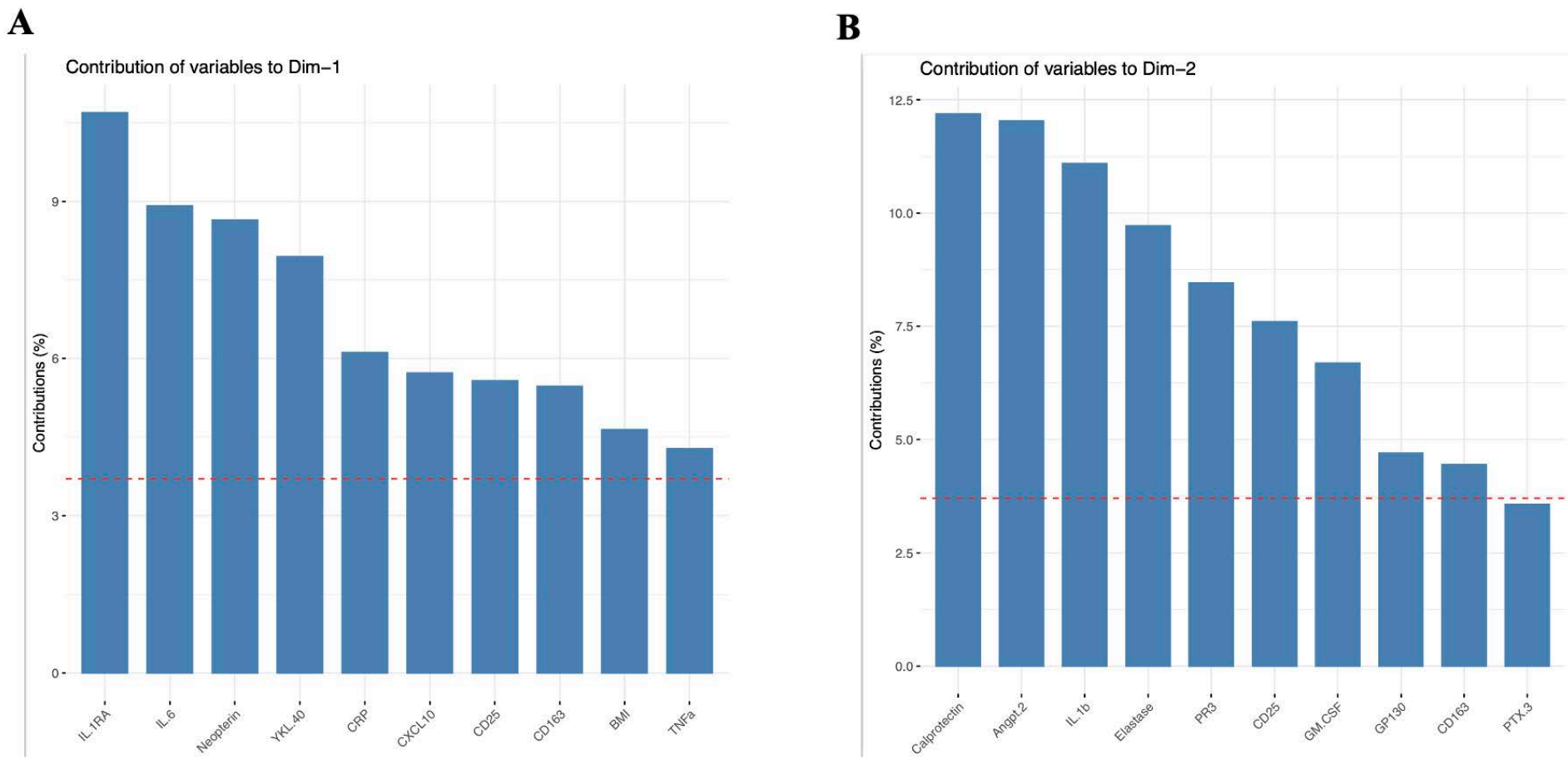
8. Supplementary Material

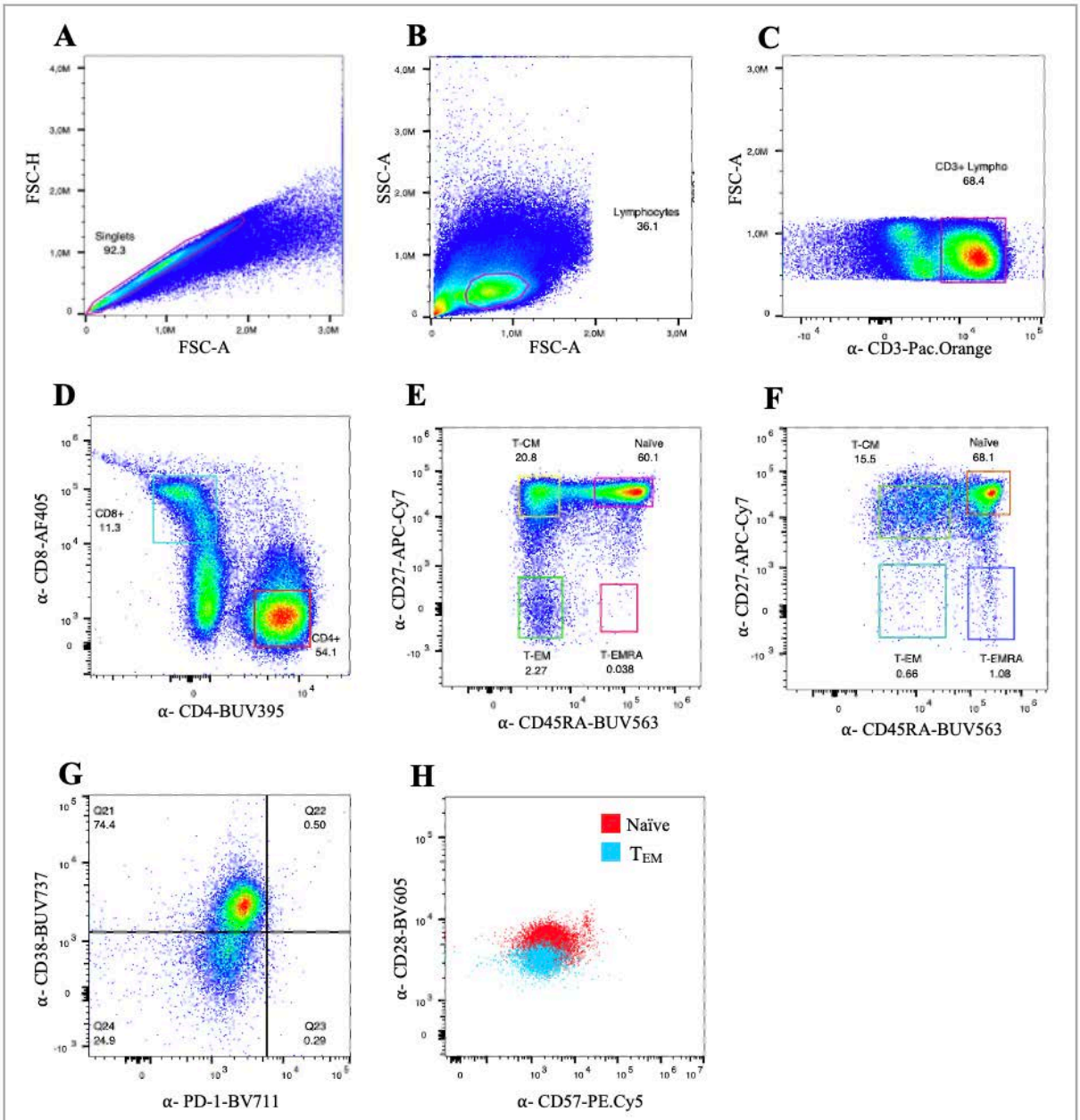
***Supplementary Table 1**. VITAL cohort average concentration of inflammation biomarkers in serum.*

	Kruskal-Wallis *p-value*	Adult (<50 years)	Pre-Elderly (50-65 years)	Elderly (>65)
***Neopterin** [nmol/L]*	$9.33x10^{-11}$	6.49 (± 3.00)	8.36^{**} (±4.07)	$11.16^{***/+++}$ (±5.83)
***IL-6** [pg/mL]*	$2.59x10^{-13}$	0.53 (± 0.39)	1.69^{***} (±3.79)	$1.94^{***/+++}$ (±4.75)
***IL-10** [pg/mL]*	0.09	0.66 (± 0.51)	0.87 (±1.44)	0.79 (±0.97)
***GM-CSF** [pg/mL]*	0.83	0.20 (± 0.12)	0.18 (±0.08)	0.19 (±0.09)
***IL-1β** [pg/mL]*	0.06	0.04 (± 0.04)	0.14 (±0.55)	0.06 (±0.23)
***TNF-α** [pg/mL]*	0.05	2.20 (± 0.72)	3.15 (±4.71)	2.37 (±1.10)
***IFN-γ** [pg/mL]*	$3.37x10^{-6}$	0.04 (±0.04)	0.09 (±0.27)	$0.08^{***/+++}$ (±0.15)
***iFABP2** [pg/mL]*	$6.39x10^{-5}$	1088 (±763)	1251^{*} (±596)	1474^{***} (±874)
***CRP** [µg/mL]*	$1.47x10^{-4}$	1.76 (± 2.96)	1.89 (±2.45)	$2.67^{***/++}$ (±3.13)
***CD14** [µg/mL]*	$1.39x10^{-5}$	1370 (± 293)	1425 (±347)	$1581^{***/+++}$ (±404)
***Calprotectin** [ng/mL]*	0.32	2186 (± 2420)	2081 (±1551)	1913 (±1161)
***IL-8** [pg/mL]*	$1.82x10^{-5}$	17.74 (± 10.91)	26.22 (±20.52)	26.01^{***} (±15.59)
***Elastase** [ng/mL]*	$6.63x10^{-4}$	207 (± 158)	265 (±239)	275^{***} (±189)
***PR3** [ng/mL]*	$1.22x10^{-4}$	39.60 (± 0.47.93)	39.36 (±38.66)	$68.03^{**/++}$ (±104.1)
***Angpt-2** [pg/mL]*	$6.09x10^{-5}$	2085 (± 1155)	2079 (±888)	$2683^{***/+++}$ (±1534)
***C5a** [ng/mL]*	0.11	21.45 (± 9.25)	23.29 (±11.59)	24.61 (±11.56)
***CCL2** [pg/mL]*	$1.52x10^{-4}$	358 (± 101)	427^{*} (±120)	488^{***} (±518)
***CD25** [pg/mL]*	$3.37x10^{-8}$	488 (± 124)	509 (±169)	$657^{***/+++}$ (±410)
***CD163** [ng/mL]*	$5.77x10^{-7}$	637 (± 353)	862^{*} (±490)	$983^{***/+}$ (±540)
***CXCL10** [pg/mL]*	$1.61x10^{-13}$	20.44 (± 8.89)	28.43^{*} (±47.97)	$31.59^{***/+++}$ (±15.5)
***GP130** [ng/mL]*	$1.24x10^{-4}$	132 (± 23)	141^{**} (±22.8)	146^{***} (±22.01)
***IL-6R** [ng/mL]*	$3.72x10^{-3}$	44.27 (± 8.28)	47.37^{**} (±8.32)	48.49^{**} (±8.40)
***IL-1Ra** [pg/mL]*	$1.31x10^{-6}$	817 (± 398)	868 (±358)	$1082^{***/+++}$ (±597)
***PTX-3** [pg/mL]*	0.28	6117 (± 3293)	5611 (±2578)	6180 (±2821)
***YKL-40** [ng/mL]*	$2.02x10^{-23}$	26.34 (± 10.29)	37.03^{*} (±23.92)	$77.45^{***/+++}$ (±61.1)

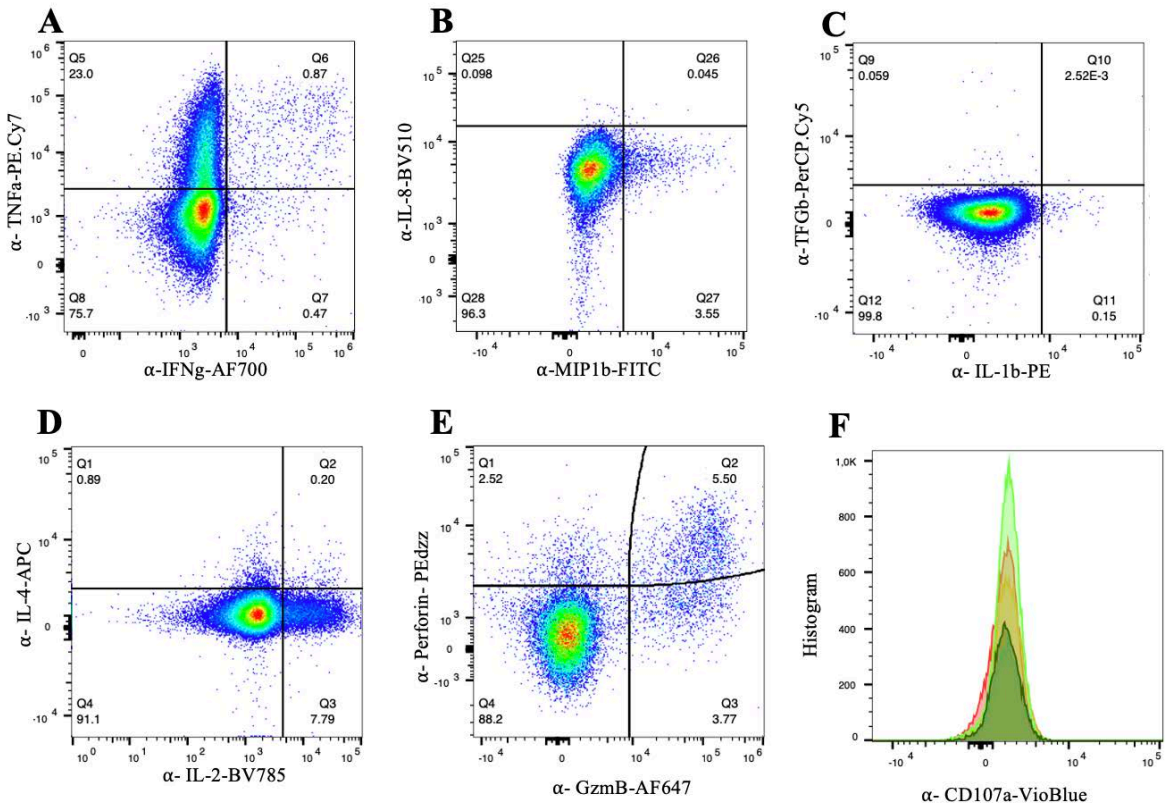
**p<0.05, **p<0.01, ***p<0.001 when compared to Adult.*
+p<0.05, ++p<0.01, +++p<0.001 when compared to Pre-Elderly.



Supplementary Figure 1. Contribution percentage of different inflammatory biomarkers to the first and second dimensions (components) of the PCA. *(A) Parameters contributing to explain the variation on the first dimension (B) Parameters contributing to explain the variation on the second dimension.*



Supplementary Figure 2. Phenotyping panel cytometry gating strategy. Gating for: (A) Single cells (B) Lymphocytes (C) CD+ Lymphocytes (D) CD8+ and CD4+ (E). Naïve, T central memory (T-CM), T effector memory (T_EM), and T terminally differentiated effector (T-EMRA) CD4+ cells (F) Naïve, T central memory (T-CM), T effector memory (T-EM), and T terminally differentiated effector (T-EMRA) CD8+ cells (G) PD-1 and CD38 (H) CD57 and CD28 for naïve (red) and T-EM (blue) lymphocytes.



Supplementary Figure 3. Function panel cytometry gating strategy. Gating for: (A) TNFα and IFNg (B) MIP1b and IL-8 (C) TGF-b and IL-1b (D) IL-2 and IL-4 (E). Perforin and Granzyme B (F) CD107a from non-stimulated (red), ConA-Stimulated (light green), SEB-stimulated (dark green), Beads-stimulated (orange).

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2021 MASTER THESIS EVALUATION FORM

The student must attach this evaluation form to his/her Master Thesis and send them in one PDF by email to mylive@univ-lyon1.fr and to his/her Academic Supervisor, with the following title: 2021_Master Thesis_YOURSURNAME. Submission deadline: May 31. The reviewers place their crosses in the boxes or write a score in the related box, as they prefer.
Evaluation deadline: June 18

Student Name:
Academic Supervisor Name:
Reviewer Name:

Criteria	<10 fail mark	10-12 pass	13-14 honors	15-16 high honors	17-18 highest honors	19-20 highest honors +
1. Max 10-pages bibliography introduction (weighted 10)						
a. Is the topic situated well within the broader scientific context?						
b. Is relevant, original and recent research cited and discussed critically?						
c. Quality of the structure of the introduction? Is specific vocabulary clearly defined and appropriately used?						
d. Formulation of the concepts, questioning & objectives						
2. Critical material and method approach (weighted 10)						
e. Definition of the applied methodology						
f. Does the Student adopt a critical view of the applied methodology?						
3. Results & discussion-perspectives (weighted 20)						
g. Are the results presented clearly?						
h. Are the results processed and analysed in a correct and critical manner?						
i. Are the results interpreted within a broader context and relevant open questions proposed for the next future?						
j. Structure and readability of the discussion and perspectives						
4. Structure of the final document (weighted 10)						
k. Is there a clear and logical structure, with coherence between the various components?						
l. Linguistic usage						
m. Quality of tables/figures and graphs						
n. Quality of the summary						
o. Display of references and footnotes						

Please kindly comment on the report below:

Reviewer date & signature:

Master thesis evaluation criteria

	Critical scientific approach		3. Results and discussion	4. Structure of the final document
	1. Introduction and objectives	2. Materials and methods		
19-20 highest honours +	a. Exceptionally good positioning of the topic within the broader scientific context; the literature is critically interpreted and processed by the student b. The literature cited is relevant, original and recent c. The structure of the introduction demonstrates exceptional insight into the topic, the introduction is structured from an original but very functional perspective d. The objectives are formulated in a very clear manner and are challenging but feasible within the time frame of the study	e. The applied methods are exceptionally well defined f. The relevance of the applied methods for achieving the objectives is demonstrated clearly; limitations of the methods are stated exceptionally well	g. The results obtained are processed exceptionally well and analysed critically, and the analysis is of an exceptionally high level h. The results are presented in an exceptionally clear and logical manner, and only the relevant results are displayed i. The discussion places the obtained results within a broader scientific context and shows exceptional insight into the background of the research j. The discussion is pleasant to read, comprehensive, yet 'to the point'	k. Exceptionally smooth and pleasurably readable text, logical and coherent structure l. Perfect linguistic usage m. Tables, figures and graphs of exceptional quality and perfectly integrated into the text n. Perfect use of references o. High-quality summary that very clearly reflects the structure and conclusions of the study
17-18 highest honours	a. Outstanding positioning of the topic within the broader scientific context, most of the cited literature is critically interpreted and processed by the student b. The cited research is relevant and recent c. The structure of the introduction demonstrates outstanding insight into the topic d. The objectives are clearly formulated and feasible within the time frame of the study	e. The applied methods are very clearly defined f. The relevance of the applied methods for achieving the objectives is demonstrated; limitations of the methods are stated very clearly	g. The results obtained are processed in an outstanding manner and analysed critically, and the analysis is of an outstanding level h. The results are presented clearly and logically, and only the relevant results are displayed i. The discussion places the obtained results within a broader scientific context and shows good insight into the background of the research j. The discussion is pleasant to read and comprehensive	k. Smoothly readable text with a logical and coherent structure l. Very good linguistic usage m. Tables, figures and graphs of very good quality and very well integrated into the text n. Very good use of references o. High-quality summary that clearly reflects the structure and conclusions of the study
15-16- high honours	a. Very good positioning of the topic within the broader scientific context; a portion of the cited literature is critically interpreted and processed by the student b. The cited research is relevant c. The structure of the introduction demonstrates very good insight into the topic d. The objectives are clearly defined	e. The applied methods are clearly defined f. The limitations of the method are discussed clearly to a certain extent	g. The results obtained are processed and analysed very well h. The results are presented clearly, but some of the results presented are not relevant i. The discussion demonstrates insight into the background of the research j. The discussion is pleasant to read	k. Easily readable text, logically structured l. Good linguistic usage m. Tables, figures and graphs of good quality and well integrated into the text n. Good use of references o. Good summary
13-14 honours	a. The topic is well situated within the broader scientific context, and the literature is interpreted critically to a limited extent by the student b. The cited research is largely relevant c. The structure of the introduction demonstrates good insight into the topic d. The objectives are formulated	e. The applied methods are present and defined to a limited extent f. The limitations of the method are discussed to a minimal extent	g. The results obtained are processed and analysed well h. The results are presented clearly enough, but not all of the presented results are relevant i. The discussion demonstrates limited insight into the background of the research j. The discussion is pleasant to read, but lacks some essential points or is not always clear	k. Easily readable text with a largely logical structure l. Occasional grammatical errors m. Tables, figures and graphs can be clearer and better integrated (more info ...) n. Good use of references o. Solid summary
10-12 pass	a. The subject is situated within the broader scientific context to a limited extent; the literature is barely interpreted by the student b. The cited research is not entirely relevant or recent c. The structure of the introduction demonstrates limited insight into the topic d. The objectives are unclear/incomplete	e. The applied methods are present but not clearly defined f. The limitations of the method are not discussed	g. The results obtained are insufficiently processed and analysed h. The results are presented incorrectly in part i. The discussion demonstrates very limited insight into the background of the research j. The discussion is difficult to read and misses essential points or is not clear	k. Text is acceptable, but not easily readable and has no clear structure l. Multiple grammatical errors m. Tables, figures and graphs can be clearer and are not well integrated into the text n. Limited use of references o. Summary does not accurately reflect the structure and conclusions of the research
<10 fail mark	a. The topic is incorrectly situated within the broader scientific context; the literature is not interpreted by the student b. The cited research is not relevant c. The structure of the introduction demonstrates very limited insight into the topic d. The objectives are not reflected accurately	e. The applied methods are not presented correctly or they are missing f. The limitations of the method are discussed incorrectly	g. The found data are not processed and analysed, or they are processed and analysed incorrectly h. The results are presented incorrectly i. The discussion demonstrates incorrect insight into the background of the research j. The discussion is very difficult to read and misses essential points or is not clear	k. Very unclear text l. Frequent grammatical errors m. Tables and figures and graphs are unclear or incorrect n. Incorrect use of references o. Summary is unclear or absent