1   **THE IMMUNOLOGICAL FACTORS PREDISPOSING TO SEVERE COVID-19 ARE ALREADY**

2   **PRESENT IN HEALTHY ELDERLY AND MEN**

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10   **Summary:** Immunological profile of severe COVID-19, characterized by altered immune cell

11   populations and inflammatory plasma proteins is intrinsically present in healthy men and the elderly.

12   Different age and sex groups show distinct seasonal responses to SARS-CoV-2.

13

14   **Short title:** Factors Predisposing to Severe COVID-19

15   **Abstract**

16   **Background:** Male sex and old age are risk factors for COVID-19 severity, but the underlying causes

17   are unknown. A possible explanation for this might be the differences in immunological profiles in

18   males and the elderly before the infection. Given the seasonal profile of COVID-19, the seasonal

19   response against SARS-CoV-2 could also be different in these groups.

20   **Methods:** The abundance of circulating proteins and immune populations associated with severe

21   COVID-19 was analyzed in 2 healthy cohorts. PBMCs of female, male, young, and old subjects in

22   different seasons of the year were stimulated with heat-inactivated SARS-CoV-2.

23   **Results:** Several T cell subsets, which are known to be depleted in severe COVID-19 patients, were

24   intrinsically less abundant in men and older individuals. Plasma proteins increasing with disease

25   severity, including HGF, IL-8, and MCP-1, were more abundant in the elderly and males. The elderly

26   produced significantly more IL-1RA and had a dysregulated IFNγ response with lower production in

27   the summer compared with young individuals.

28   **Conclusions:** The immune characteristics of severe COVID-19, described by a differential abundance

29   of immune cells and circulating inflammatory proteins, are intrinsically present in healthy men and

30   the elderly. This might explain the susceptibility of men and the elderly to SARS-CoV-2 infection.

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32   **Word Count:**200

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34   **Keywords:** COVID-19, aging, sex differences, seasonality, SARS-CoV-2

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INTRODUCTION

Having emerged in China in December 2019, the coronavirus disease (COVID-19) caused by severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) has become a major health crisis. As of April 2021, SARS-CoV-2 has led to over 135 million infections and more than 3 million deaths worldwide [1].

The most vulnerable groups are people older than 70 years old and adults with underlying health conditions such as chronic respiratory problems and diabetes [2]. While age is the strongest predictor of death from COVID-19, the sharp increase in fatality after 50 years of age is more critical in men [3, 4]. Association of male sex with higher mortality has been consistently reported in different populations [5-7].

The factors underlying the impact of age and sex on the susceptibility to severe COVID-19 are, however, incompletely understood. Most studies to date have focused on the differences between either young and old or men and women during the disease process. A recent study has shown that male COVID-19 patients had higher circulating concentrations of cytokines such as IL-8 and IL-18, as well as a greater abundance of non-classical monocytes [8]. In contrast, a higher degree of T cell activation was observed in females than males during SARS-CoV-2 infection.

We previously analyzed 269 circulating proteins to identify COVID-19 severity markers by comparing patients in the intensive care unit (ICU) to patients who do not require ICU admission [9]. Several cytokines and chemokines such as IL-8 and monocyte chemoattractant protein-3 (MCP-3), and growth factors, e.g., hepatocyte growth factor (HGF), were increased in ICU patients, whereas stem cell factor (SCF) and several TNF-family proteins, e.g., TNF-related activation-induced cytokine (TRANCE) were decreased in ICU patients. Besides, frequently reported severe COVID-19 characteristics include elevated TNFα, IL-6, MCP-1, IP-10, and IL-10 concentrations, lower numbers and activity of T, B, and NK cells, lower antigen presentation, and downregulated type I interferon signaling [10-13]. Changes in cell populations and plasma proteins related to COVID-19 severity are

61   also summarized in Supp. Table 1. However, it is unknown whether such differences are induced by

62   the disease severity itself, or the potential to respond differently was already present in the healthy

63   steady-state condition.

64   In this study, we analyzed some of the immune cell populations and circulating proteins linked to

65   COVID-19 severity (Supp. Table 2) in two Dutch cohorts of healthy individuals. Our reasoning for

66   choosing these parameters is based on the type of data available from the two cohorts. We

67   investigated if healthy men and individuals over 50 years old already have an immunological profile

68   that predisposes them to severe COVID-19 progression upon SARS-CoV-2 infection. Although the

69   pandemic's seasonal character is not completely clear, several studies reported links to temperature

70   and humidity [14, 15]. Therefore, we also hypothesized that immunological differences induced by

71   the seasons could influence the disease outcome. Hence, we investigated SARS-CoV-2-induced

72   immune responses in healthy individuals *in vitro* at different time points of a year using cryo-

73   preserved PBMCs from one of the cohorts. This study provides new insights into how age, sex, and

74   seasons influence COVID-19 response (Figure 1).

75

76   **MATERIALS & METHODS**

77   ***Study Cohorts***

78   534 healthy individuals of Western European origin were included in Cohort 1 (500 Functional

79   Genomics Project, see www.humanfunctionalgenomics.org) at the Radboud University Medical

80   Center between August 2013 and December 2014. 45 volunteers were initially excluded due to

81   medication use and chronic diseases, while 37 participants were later excluded from the analysis

82   because one or more measurements were unavailable. Data of 452 participants, 229 females and

83   223 males with age ranges of 18-70 and 18-75, respectively, were used for analysis.

84   324 healthy individuals of Western European origin, Cohort 2, were included from April 2017 until

85   June 2018 at the Radboud University Medical Center. 183 participants were female, and 141 were

86   male with age ranges of 18-62 and 18-71, respectively. This cohort served as a validation cohort for

87   proteomics.

88   Both studies were approved by the Arnhem-Nijmegen Medical Ethical Committee (NL42561.091.12

89   and NL58553.091.16). Inclusion and experimentation procedures were conducted according to the

90   principles of the Declaration of Helsinki. Written informed consent was obtained from all volunteers

91   before sample collection.

92   ***Proteomics***

93   Plasma proteins were measured using the Proximity Extension Assay (PEA) by Olink Proteomics

94   (Uppsala, Sweden). The Olink Inflammation Panel consisting of 92 inflammation-related biomarkers

95   was measured. This assay provides relative protein quantification expressed as normalized protein

96   expression (NPX) values on a log2 scale. The proteins for which the missing data frequency was over

97   20 % were excluded from the analysis. The remaining data under the detection limit was replaced

98   with the lower limit of detection for each protein. Measurements were normalized according to

99   inter-plate controls.

100   ***Flow Cytometry***

101   Immune cell types in Cohort 1 were measured from whole blood by 10-color flow cytometry with

102   Navios flow cytometer (Beckman Coulter, CA, USA). Staining and gating strategies were previously

103   described in detail by Aguirre-Gamboa et al. [16].

104   ***In vitro Stimulations and Cytokine Measurements***

105   From the 452 individuals in Cohort 1, a sub-cohort of 50 people were asked to donate blood at 4

106   different time points in a year. Peripheral blood mononuclear cells (PBMCs) were collected and cryo-

107   preserved between February 2016 and February 2017 to assess the seasonality of immune

108   responses. From those 50, we selected 20 individuals considering the optimal age and sex matching

109   of young-old and male-female comparisons. Therefore, cells isolated from 5 young males, 5 old

110   males, 5 young females, and 5 old females were included for an *in vitro* study assessing seasonality's

111   impact on the cytokine responses to SARS-CoV-2. Cohort demographics are shown in Supp. Table 3.

112   Upon thawing, PBMCs were stimulated with heat-inactivated SARS-CoV-2 at a concentration of

113   3.3×10^3 TCID50/mL and heat-inactivated Influenza A H1N1 (California strain) at a concentration of

114   3.3×10^5/mL for 24 hours and 5 days. Cytokine concentrations in the supernatants were measured

115   with DuoSet® ELISA kits (R&D Systems, MN, USA) according to the manufacturer's protocols.

116   ***Statistical Analyses***

117   Statistical analyses were performed unsing R 3.6.1 (www.R-project.org) and GraphPad Prism 8

118   (GraphPad Software Inc., CA, USA). After adjusting the data for the covariate sex using linear

119   regression, correlation of cell numbers or protein levels with age was done using Spearman's rank-

120   order correlation (Figures 2 and 4). Correction for sex was not applied in the heatmaps where two

121   sexes were analyzed separately. After adjusting the data for the covariate age, differential protein

122   expression or cell numbers between males and females was tested using the Mann-Whitney test

123   (Figures 3 and 5). For box plots comparing different age groups, the two sexes, or four seasons, the

124   Mann-Whitney test was used between any two groups. The Benjamini-Hochberg procedure was

125   employed to correct multiple testing errors for the heatmaps and volcano plots. False discovery rate

126   (FDR)-adjusted p-values smaller than 0.05 were considered statistically significant.

127

128   **RESULTS**

129   ***Age-dependent Changes in Immune Cell Populations Linked to COVID-19 Severity***

130   Various immune cell sub-types were correlated with age in Cohort 1. Non-classical monocytes

131   increased with advancing age in both sexes, but intermediate and classical monocyte numbers were

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132   not significantly correlated with age. CD56^{bright} NK population decreased with age in females while

133   other NK cell types remained unchanged (Figure 2). Previously, these results were partly reported by

134   our group [16].

135   Total T cell count was negatively correlated with age, indicating the lymphopenia already

136   experienced by the elderly, even in health, is likely to predispose to COVID-19 severity. Naïve T cells,

137   especially CD8^{+}, exhibited the most striking age-dependent decline in both sexes. Naïve regulatory T

138   cells (Tregs) also decreased considerably with age in males, whereas memory Treg numbers were

139   elevated. Total and naïve B cell numbers were negatively correlated with age. Plasmablasts similarly

140   declined significantly in females. No significant age-related changes were observed among the other

141   B cell sub-types.

142   Overall, these results demonstrate that naïve CD4^{+}, CD8^{+}, Treg, and B cell pools, as well as CD56^{bright}

143   NK cells, which are all depleted in severe COVID-19, also decrease with age. The differences are clear

144   even from the age of 50.

145

146   ***Sex-dependent Patterns of Immune Cell Populations Linked to COVID-19 Severity***

147   Next, we investigated the differential abundance of the same immune cell populations influencing

148   COVID-19 severity in females vs. males. Only CD8^{+} effector memory T cells were significantly more

149   abundant in males (Figure 3). Almost all cell types, including neutrophils, naïve CD4^{+} and CD8^{+} T cells,

150   memory T cells, class-switched memory B cell, and CD56^{bright} NK cell counts, were significantly higher

151   in females. The T cell types and CD56^{bright} NK cells, which are depleted in severe COVID-19 and

152   elderly healthy people, are also apparently less abundant in males.

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154   ***Age-dependent Changes in Immune Mediator Proteins Linked to COVID-19 Severity***

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155   We selected 28 proteins whose plasma concentrations have been associated with severe COVID-19

156   and correlated them with age in healthy cohorts. Circulating IL-6 concentrations increased with age

157   in healthy women, while IL-18 concentrations were higher in healthy men with advancing age in

158   Cohort 1 (Figure 4). IL-8 concentrations positively correlated with old age in both females and males;

159   however, it was only significant when sexes were combined. Among investigated chemokines, only

160   MCP-1 was positively correlated with age in females in Cohort 2.

161   TNF-family proteins in plasma also changed with increasing age: TNF and TNFB concentrations were

162   significantly lower in the circulation of older men in Cohort 1 and 2, respectively. TRANCE sharply

163   declined in older individuals, more strikingly in males, while TWEAK was positively correlated only in

164   females with advancing age. Moreover, aging in males was associated with elevated osteoprotegerin

165   (OPG) concentrations, and HGF concentrations exhibited a considerable age-dependent increase in

166   females in both cohorts.

167   In summary, several proteins in plasma that are increased in severe COVID-19 patients, such as IL-6,

168   IL-8, IL-18, MCP-1, OPG, and HGF, are more abundant in healthy elderly compared to young

169   individuals. Furthermore, proteins that are lower in severe COVID-19, e.g., TRANCE, decline with age.

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171   ***Sex-dependent Patterns of Immune Mediator Proteins Linked to COVID-19 Severity***

172   We also compared plasma protein concentrations between sexes. It must be noted that Koeken et

173   al. previously reported that males and females of Cohort 2 exhibit differences in baseline levels of

174   many inflammatory markers [17]. Here we provide a more detailed analysis of COVID-19-related

175   proteins among those in Cohorts 1 and 2. We observed a similar sex-dependent trend in both

176   cohorts (Figure 5). Only OPG and colony-stimulating factor-1 (CSF-1), related to severe COVID-19,

177   were significantly higher in women. On the other hand, plasma concentrations of other severity

178   markers such as IL-8, IL-18, MCP-1, MCP-2, CCL3, and CCL4 were all higher in men. Furthermore,

179   TRAIL, TWEAK, and TRANCE, which are all lower in COVID-19 patients in ICU, were more abundant in

180   males [9]. Males exhibited more anti-inflammatory proteins, e.g., PD-L1 and IL-10. Growth factors

181   HGF and SCF were also more abundant in male plasma.

182   These analyses show that most of the inflammatory mediators playing a role in infection severity are

183   already higher in the circulation of healthy men.

184   We hypothesized that circulating inflammatory proteins could be related to impaired cytokine

185   response against the virus. Therefore, we correlated SARS-CoV-2-induced *in vitro* cytokine

186   productions with baseline circulating protein concentrations in a sub-cohort of Cohort 1. Indeed,

187   PBMCs of the individuals with higher baseline plasma levels of MCP-2 and IL-8 produced more IL-1RA

188   against SARS-CoV-2 *in vitro* (Supp. Figure 1). Furthermore, MCP-1 was negatively correlated with

189   IFNγ production. The data indicate that higher baseline plasma concentrations of MCP-2, IL-8, and

190   MCP-1 are associated with the inability to produce an optimal defense against SARS-CoV-2 infection.

191

192   ***Sex, Age, and Season as Influencing Factors of Immune Response Against SARS-CoV-2***

193   Next, we investigated the impact of seasonality on the SARS-CoV-2-induced immune response and

194   assessed the contribution of age and sex. To this end, we selected 20 individuals from Cohort 1, for

195   which cryo-preserved cells collected at 4 roughly equidistant time points in one year were available.

196   We stimulated their PBMCs with heat-inactivated SARS-CoV-2 and influenza A H1N1.

197   We found that cytokine production upon SARS-CoV-2 stimulation did not substantially vary during

198   the year, considering all 20 individuals (Supp. Figure 2). However, SARS-CoV-2-induced cytokine

199   production did differ for different age groups and sexes throughout the year. Cytokines of the IL-1

200   biological pathway were higher in the elderly: IL-1β production tended to be greater in the elderly

201   than in young individuals (Figure 6A), while SARS-CoV-2 induced more IL-1RA all-year-round in the

202   old individuals(Figure 6B). Interestingly, IFNγ production upon stimulation with SARS-CoV-2 had a

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203   different seasonal profile in the young and elderly: young individuals produced more IFNγ in the

204   summer and fall (Figure 6C). Remarkably, the elderly did not display this seasonal effect, with low

205   IFNγ production throughout the year. TNFα and IL-6 productions upon stimulation were similar in

206   the young and the elderly (Supp. Figure 3A-B). In addition, IL-1β production in response to SARS-

207   CoV-2 in spring and summer was higher in females; however, the average yearly response failed to

208   reach statistical significance (Figure 6D). IL-1RA and IFNγ production in males and females were

209   comparable (Figure 6E-F).

210   Stimulating PBMCs using another RNA virus, influenza H1N1, resulted in a similar pattern to SARS-

211   CoV-2 stimulation regarding the age and sex effects. The elderly tended to produce more IL-1β and

212   IL-1RA, while young individuals could produce higher IFNγ amounts upon influenza stimulation

213   (Supp. Figure 4). Similar amounts of IL-1β, IL-1RA, and IFNγ were induced in males and females on

214   average, with few exceptions (Supp. Figure 4D-F).

215   These data show that individuals of distinct ages and sexes respond differently to SARS-CoV-2

216   infection depending on the seasons of the year.

217

218   **DISCUSSION**

219   Old age and male sex are important risk factors for COVID-19 severity. Several studies have

220   investigated whether immune responses during SARS-CoV-2 infection are influenced by

221   demographic factors such as age and sex [18, 19]. Although they identified immune profiles

222   associated with severe COVID-19, they could not assess whether these were secondarily induced by

223   the disease, or due to *a-priori* immune differences between different groups. In the present study,

224   we show that the immune characteristics associated with severe COVID-19, such as specific changes

225   in cell populations and circulating inflammatory proteins, are already present in healthy elderly and

226   men. Interestingly, while the season did not impact the immune response to SARS-CoV-2 stimulation

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227 in the entire group, there was a clear difference in the responses between the young and old. Young

228 individuals, but not the elderly, improve their IFNγ responses to SARS-CoV-2 during the summer.

229 COVID-19 progression to a severe clinical picture is related to the depletion of several immune cell

230 types, including naïve CD4$^+$ and CD8$^+$, and CD56^{high} NK cells. Our study demonstrates the age-related

231 decline of these cells in healthy individuals even before the infection, which likely contributes to

232 their incapacity to eliminate the virus. These data are supported by studies suggesting that some of

233 these cell types are scarcer in uninfected elderly and males [20, 21]. Naïve B lymphocyte numbers

234 were also reduced with advanced age, which would undermine the development of adaptive

235 immunity and antibody production upon infection [22]. The aging process does not only alter cell

236 numbers, but also the functions (Figure 6C) [23]. All these might cumulatively disrupt the response

237 against SARS-CoV-2 infection.

238 We showed that one of the striking differences in immune cell types between males and females

239 was the number of CD4$^+$ T cells. Although we cannot rule out that significant differences in cell

240 populations might not determine the disease severity, SARS-CoV-2-specific CD4$^+$ T cells were

241 strongly linked with milder COVID-19, unlike antibodies and CD8$^+$ T cell numbers [24]. Fast induction

242 of CD4$^+$ T cells was related to a milder disease, while defects in inducing SARS-CoV-2-specific CD4$^+$ T

243 cells were associated with severe or fatal COVID-19.

244 An exaggerated systemic inflammation has been associated with severe COVID-19, mirrored by high

245 circulating concentrations of pro-inflammatory mediators [25]. Among those, IL-8, IL-18, and MCP-1

246 have been frequently reported, and the first two characterize the immune response of men with a

247 severe outcome [8]. Notably, their concentrations are already higher in both men and the elderly in

248 our healthy cohorts. Another protein displaying the same pattern is HGF, acting on epithelial and T

249 cells promoting migration [26], which has higher concentrations in the circulation of severe patients

250 [27]. The circulating HGF concentrations are higher in men, but also increases with age in women.

251   Chemokines are critical inflammatory mediators, and MCP-2, CCL3, CCL4, CCL19, and CXCL10 are all

252   more abundant in males. ENRAGE (S100A12), produced by neutrophils and monocytes, is also higher

253   in healthy males than females. Monocytes expressing high S100A12 and IL-8 are linked to COVID-19

254   severity [28]. Additionally, anti-inflammatory proteins IL-10 and PD-L1 are elevated in healthy males

255   and severe COVID-19. Initially considered a negative feedback mechanism for infection-induced

256   inflammation, there are arguments suggesting these proteins as biomarkers of immune exhaustion,

257   which is likely to play a substantial role in the pathophysiology of COVID-19 [29, 30]. Notably, the

258   association of early IL-10 production with COVID-19 severity supports this idea [31].

259   Severity markers increasing with old age, but not affected by sex, include IL-6 and OPG. IL-6 secreted

260   by hyperactive monocytes contributes to low HLA-DR expression and lymphopenia in severe COVID-

261   19 [32]. TNF-family cytokine receptor OPG, abundant in ICU patients, increases with old age in our

262   healthy cohorts [9]. High OPG concentrations in females are likely due to estrogen's effects

263   promoting OPG expression to inhibit bone resorption [33]. Another TNF-family member, TRANCE

264   (RANKL), which is lower in severe COVID-19 cases, also declines with advancing age in healthy

265   individuals. T cells are one of the primary TRANCE sources, which might explain its scarcity in the

266   elderly and severe COVID-19 patients with lymphopenia [34].

267   Of note, concentrations of circulating IL-7 and IFNγ, which are increased in severe COVID-19, are

268   similar in men and women. Therefore, T cell numbers being higher in women is unlikely due to IL-7-

269   induced lymphopoiesis, whereas higher T cell numbers do not necessarily lead to more circulating

270   IFNγ. An overview of the age- and sex-dependent immune profiles in healthy individuals potentially

271   predisposing to severe COVID-19 upon infection is provided in Figure 7.

272   Environmental factors are also known to affect immune responses. Certain infections such as

273   influenza follow a seasonal pattern [35], and the evolution of the pandemic last year also suggested

274   that COVID-19 incidence might follow a seasonal variation [14]. Therefore, we questioned whether

275   this might be due to seasonal changes in the immune response to the virus. In the entire group, we

276   found no clear seasonal response against SARS-CoV-2, although this could be due to the limited

277   sample size. Interestingly, young individuals improve their IFNγ response to SARS-CoV-2 during the

278   summer, while the elderly do not. Further research with larger cohorts is required to validate

279   seasonality's full impact on anti-SARS-CoV-2 host defense.

280   Our results indicate that the immune response upon *in vitro* SARS-CoV-2 stimulation varies

281   depending on age and sex. The response of the elderly is characterized by low IFNγ and elevated IL-

282   1RA production. IFNγ is crucial for an effective response of T- and NK cells to viral infections, and its

283   deficiency is associated with severe COVID-19 [36]. Depleted NK and T cell pools might explain why

284   the elderly are less capable of producing IFNγ upon SARS-CoV-2 stimulation. Poor IFNγ response,

285   especially in the fall, might put the elderly at higher risk for severe COVID-19. Men and women

286   produce comparable amounts of IFNγ, although the T cell numbers are higher in women. Other roles

287   of T cells besides IFNγ production may contribute to the better prognosis of women with COVID-19.

288   One important point is that these defects are present in the whole population, not at the individual

289   level: while the elderly as a group have lower immune responses, there are certainly aged individuals

290   who have effective immune reaction. This inter-individual variability could be the reason why some

291   elderly or some men have good responses against SARS-CoV-2 and develop only mild disease.

292   In COVID-19, an overproduction of pro-inflammatory cytokines contributes to the pathophysiology

293   late in the disease [37]. On the other hand, cytokines such as IL-1β might also be crucial for an early

294   anti-viral response. The deficiency of IL-1β or its receptor causes higher viral load and mortality in

295   murine models [38]. Furthermore, genetic variants in *IL1B* contribute to influenza susceptibility in

296   humans [39]. We observed higher IL-1β production in women in response to SARS-CoV-2 *in vitro*,

297   arguing that an initial potent anti-viral defense is essential to prevent severe disease. Moreover, IL-

298   1RA is an antagonist of IL-1 bioactivity, helping prevent excessive inflammation [40]. However, early

299   IL-1RA production in patients is associated with COVID-19 severity [31], while our finding of higher

300   IL-1RA production in the elderly suggests that IL-1RA might be hindering their ability to mount an

301   optimal immune response against SARS-CoV-2. Alternatively, high IL-1RA production with increasing

302   age might mirror the general inflammatory profile of elderly individuals.

303   In conclusion, our findings shed light on the immunological factors that might explain why men and

304   the elderly have a higher risk of developing severe COVID-19. These results also emphasize the

305   importance of the IL1β/IL-1RA axis and IFNγ in anti-SARS-CoV-2 response. We propose that

306   intrinsically different immune characteristics, including plasma inflammatory mediators and immune

307   cell populations, in healthy people would influence their immune response upon SARS-CoV-2

308   infection and the severity of the disease. Results of this study inform prophylactic and therapeutic

309   efforts.

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315

316   **Conflict of Interest**

317   The authors declare no conflict of interest.

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417

Figures

Figure 1. (Left) Potential COVID-19 risk factors investigated in this study. (Right) Frequently reported immunological characteristics of severe COVID-19 patients. Downward arrows depict a decline, while upward arrow represents an increase.

Figure 2. (Left) Heatmaps depicting Spearman correlation of age with cell numbers in Cohort 1. Red depicts positive correlation while blue depicts negative correlation. Data were controlled for the covariate sex when analyzing the whole cohort. (Right) Exemplary bar plots of selected cell populations in individuals aged less than 50, more than 50, and more than 60. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$. ⚥ whole cohort, ♀ females (n = 229), ♂ males (n = 223).

Figure 3. (Top) Volcano plot depicting differential numbers of cell populations depending on sex in Cohort 1. Significant results are depicted in red. Data were controlled for the covariate age. (Bottom) Exemplary bar plots of selected cell populations in females and males. *$p \leq 0.05$, **$p \leq 0.01$.

Figure 4. (Left) Heatmaps depicting Spearman correlation of age with plasma levels of proteins linked to COVID-19 severity. Red depicts positive correlation while blue depicts negative correlation. Data were controlled for the covariate sex when analyzing the whole cohort. (Right) Exemplary bar plots of plasma levels of selected proteins in individuals aged less than 50, more than 50, and more than 60. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$. ⚥ whole cohort, ♀ females ($n_{Cohort1}$ = 229, $n_{Cohort2}$ = 183), ♂ males ($n_{Cohort1}$ = 223, $n_{Cohort2}$ = 141).

Figure 5. (Top) Volcano plots depicting differential plasma levels of proteins linked to COVID-19 severity depending on sex. Significant results are depicted in red. Data were controlled for the covariate age. (Bottom) Exemplary bar plots of selected proteins in females and males. ****$p \leq 0.0001$.

440 **Figure 6.** Cytokine responses against heat-inactivated SARS-CoV-2 in healthy individuals. Each panel's

441 left graphs show the yearly average production, while the right graphs demonstrate cytokine

442 production in every season. Responses were compared between young and old individuals (A-C) and

443 between males and males (D-F). IL-1β and IL-1RA cytokine levels were measured after 24 hours,

444 while IFNγ was measured after 5 days. *p ≤ 0.05, **p ≤ 0.01. n=8-10. Error bars depict the standard

445 error of the mean (SEM).

446 **Figure 7.** Age- and sex-dependent factors in healthy individuals that are in-line with the severe

447 COVID-19 phenotype.

448

Risk Factors

Age
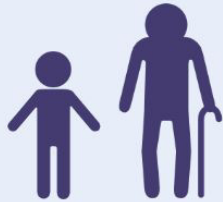
Sex

Season
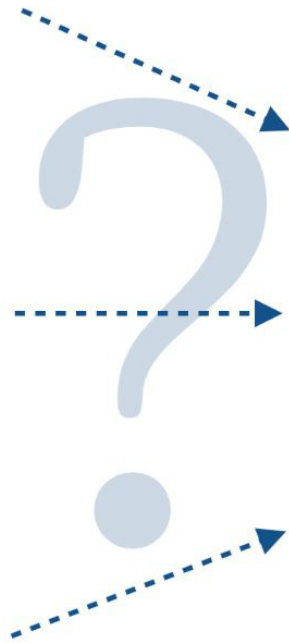
Severe COVID-19 Immune Profile
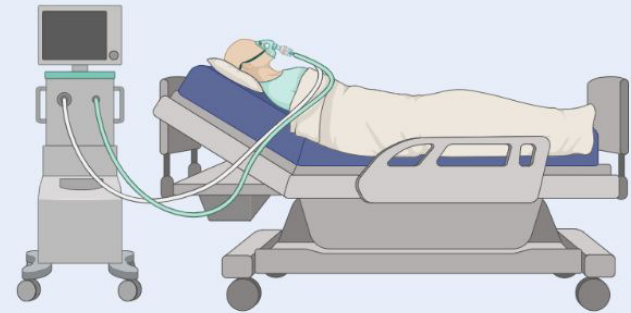
Innate immunity
↓ Antigen presentation
Interferon signaling

Adaptive immunity
↓ Lymphocyte numbers,
activation, and cytotoxicity

Circulation
↑ Inflammatory cytokines
and chemokines

CD4_T_Cells

CD4_Central_Memory   Memory_Tregs
                       T_Cells   CD8_Effector_Memory
Class_Switched_Memory_B_Cells
                       CD4_Naive   Leukocytes
Plasmablasts
                                     Neutrophils
               Naive_T_Cells
                       Lymphocytes
                              NKT_Cells
        CD8_Naive   Memory_T_Cells   CD8_Central_Memory

NK_CD56bright
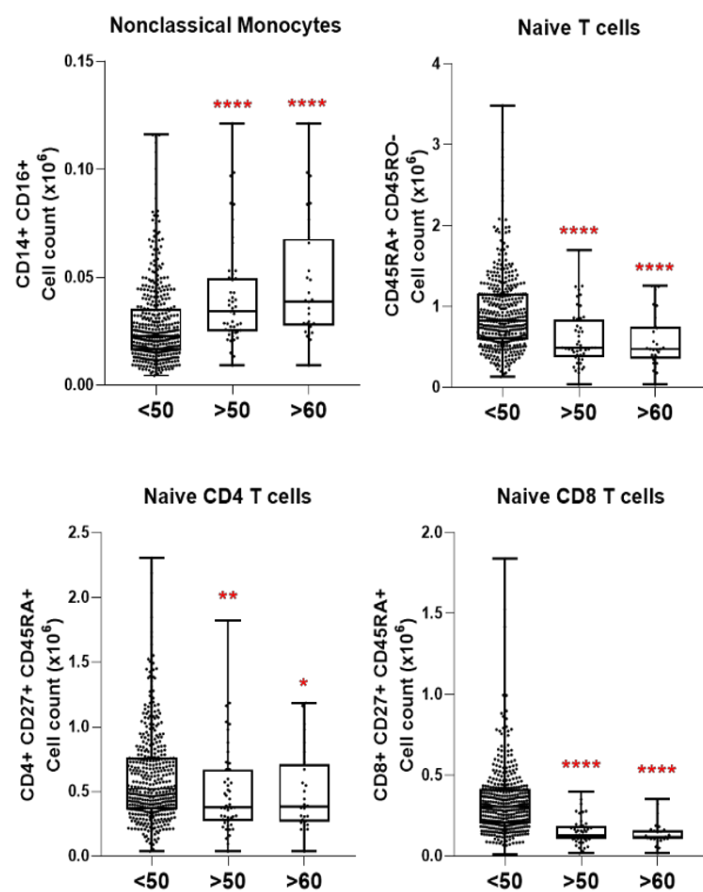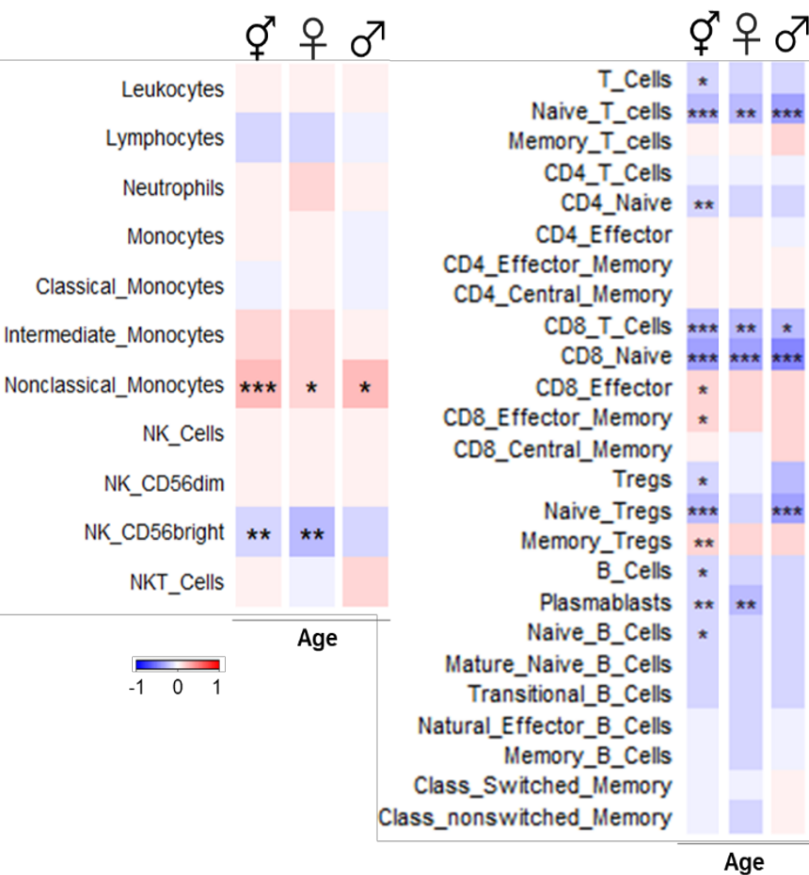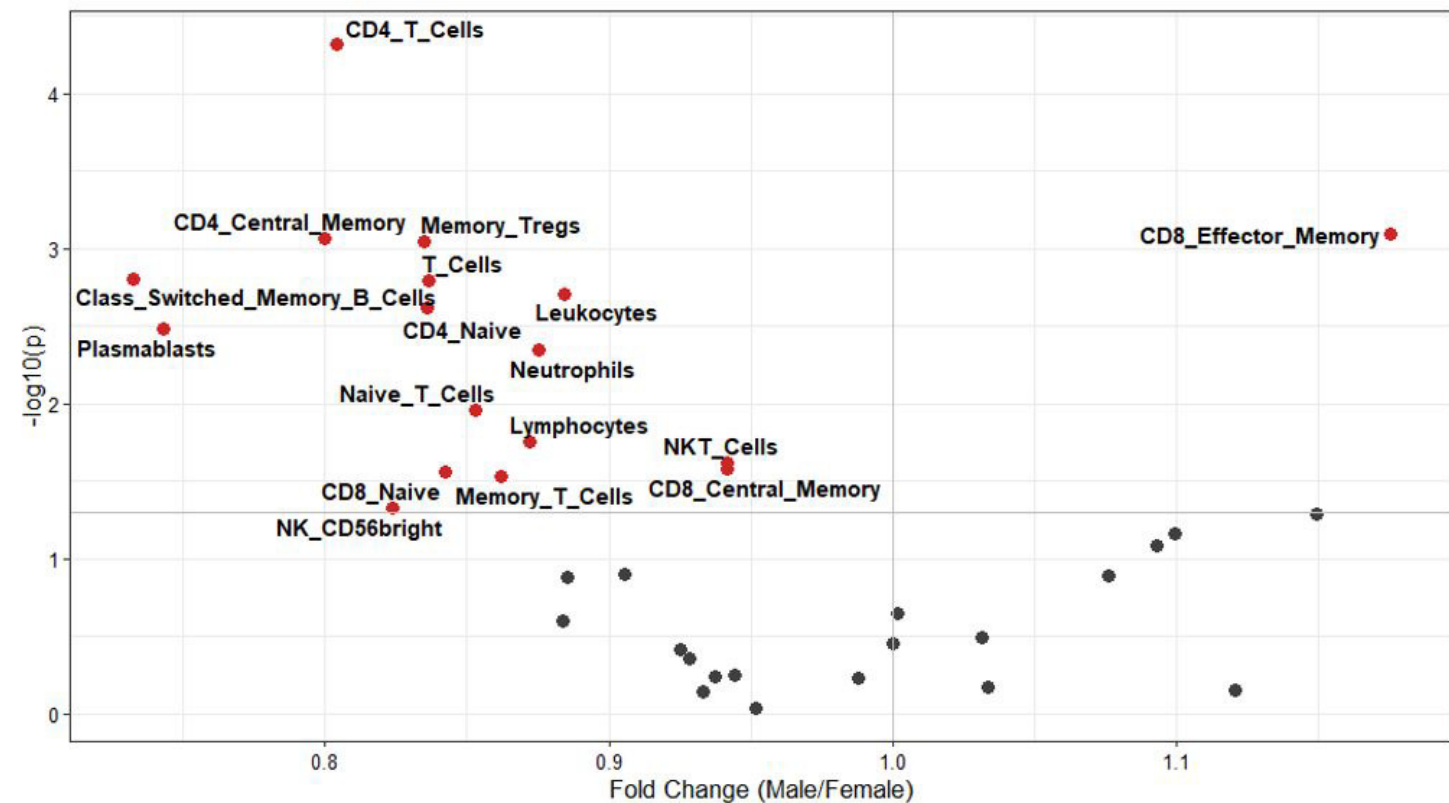
CD45+ Leukocytes
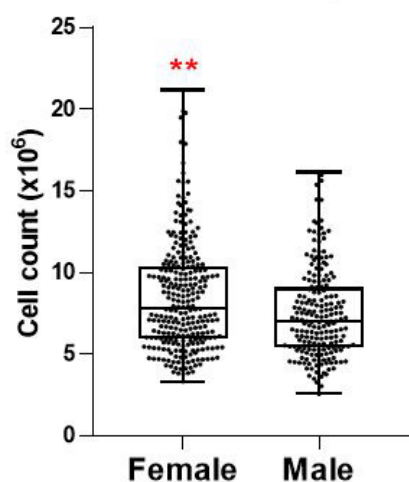
Neutrophils
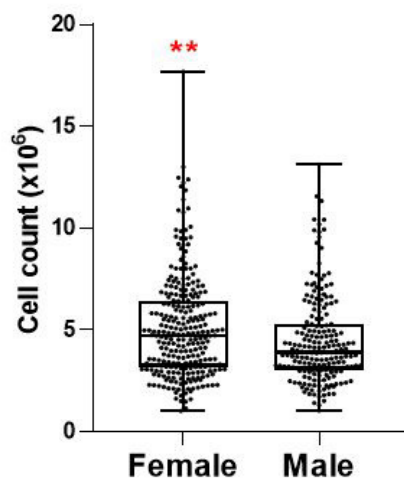
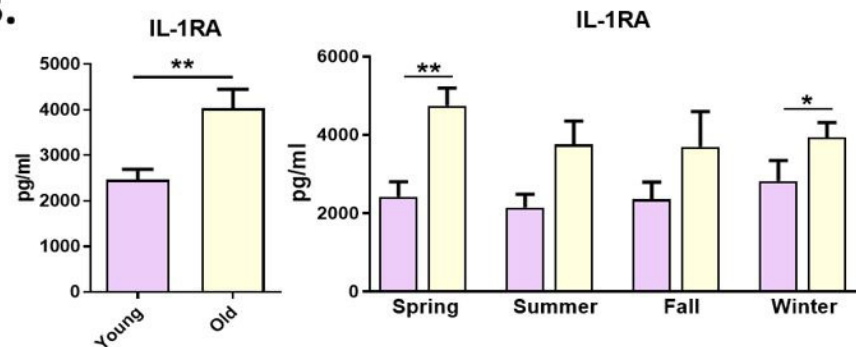**Naive T cells**
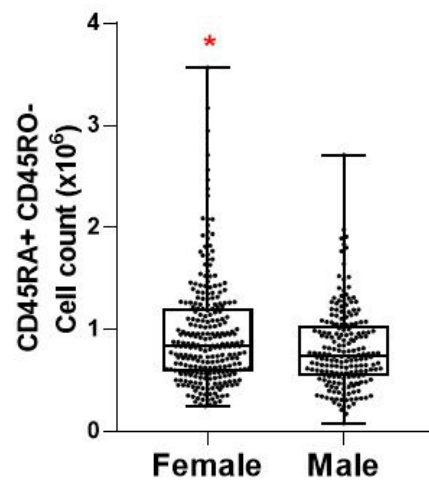
Naive CD4 T cells
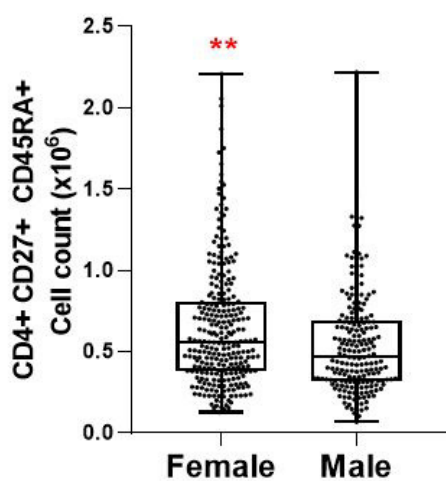
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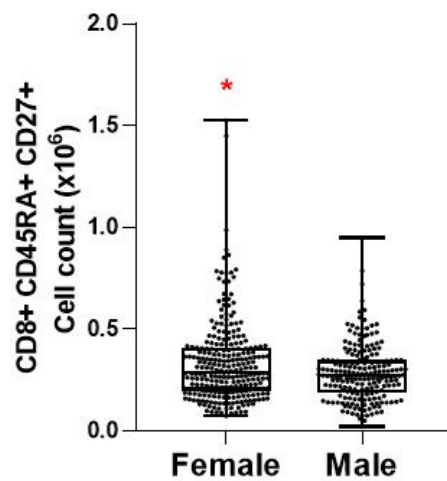
Central Memory CD4 cells
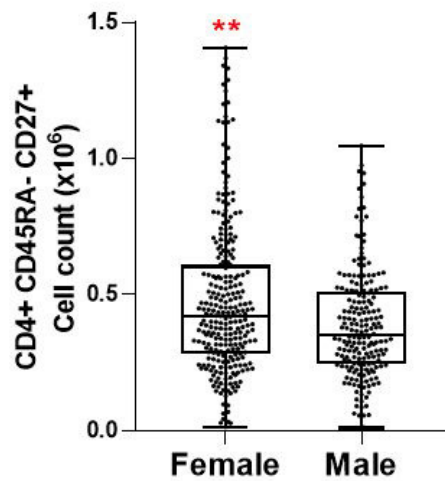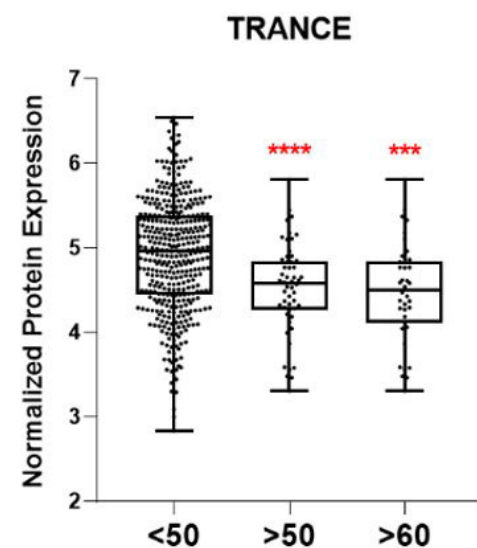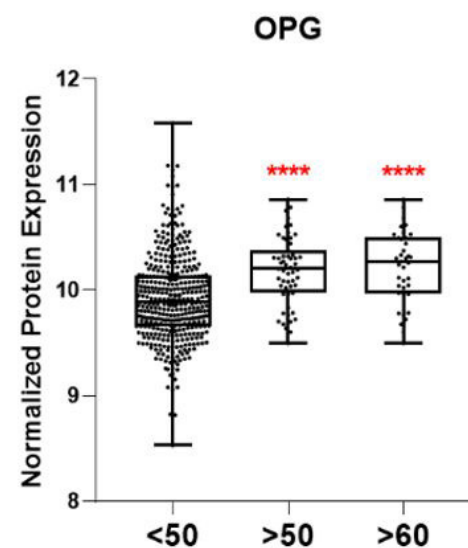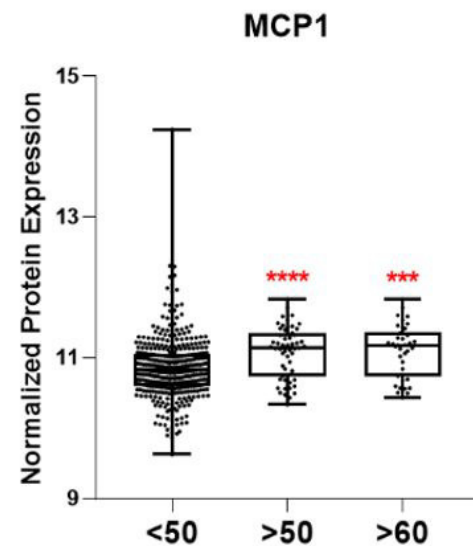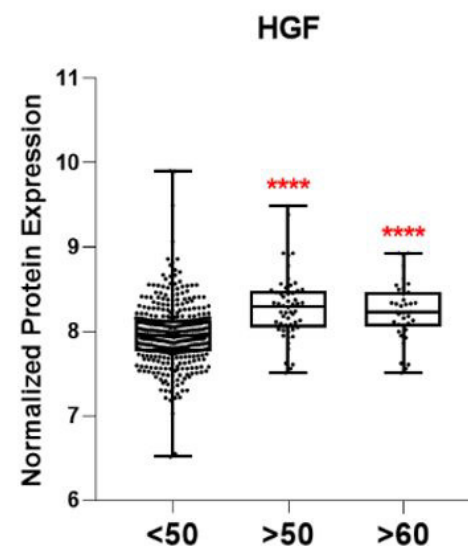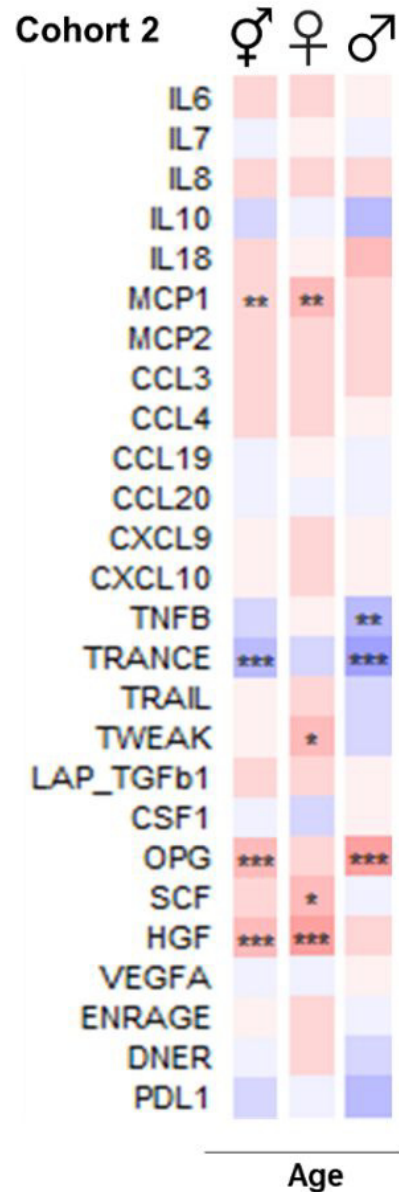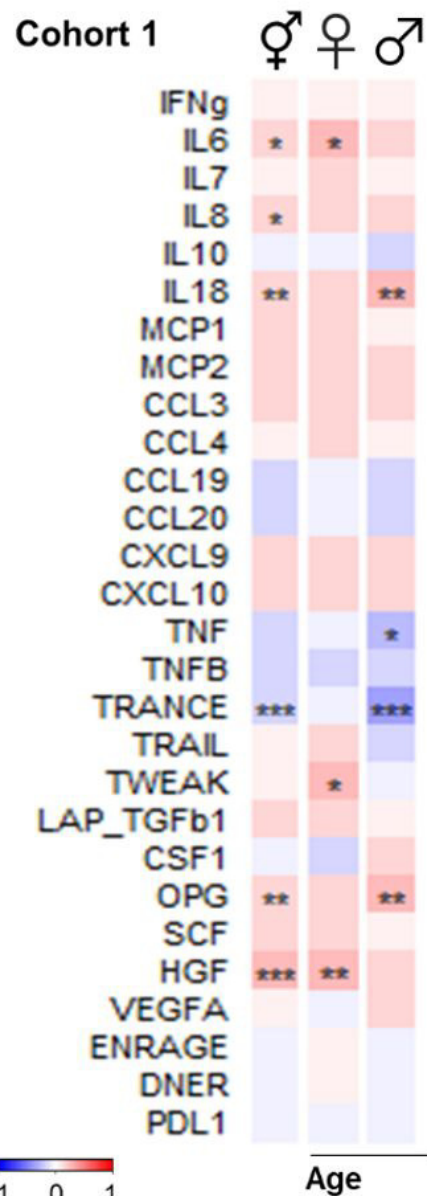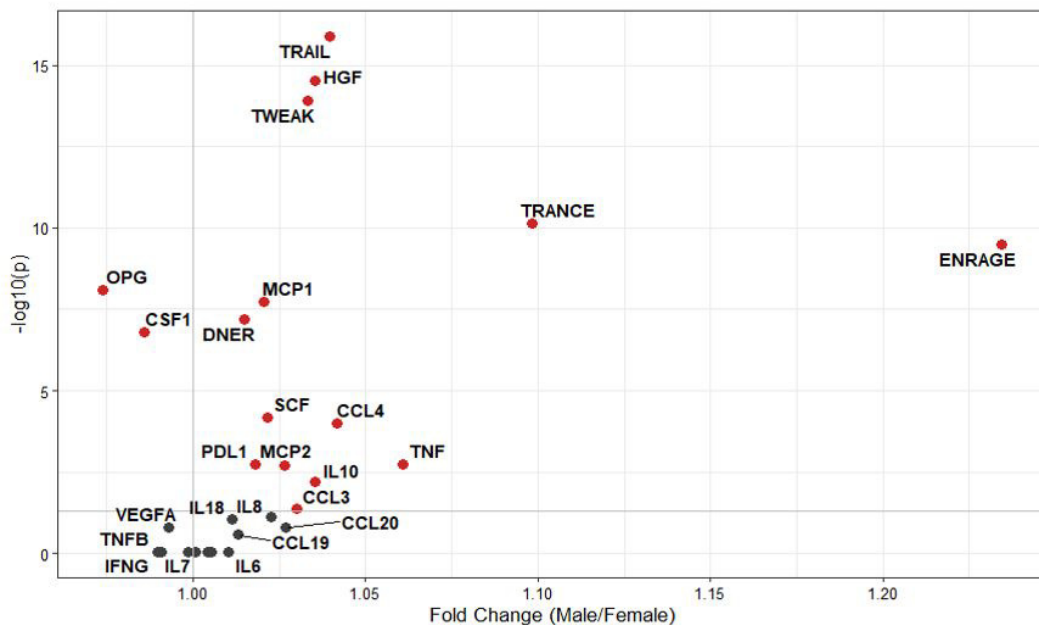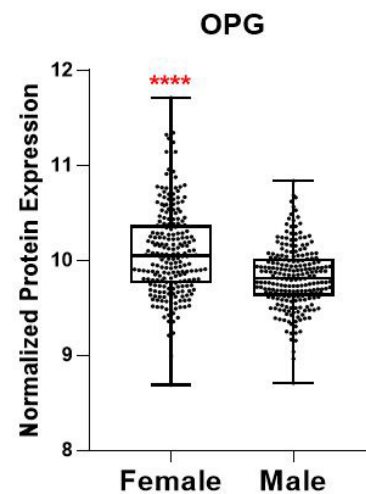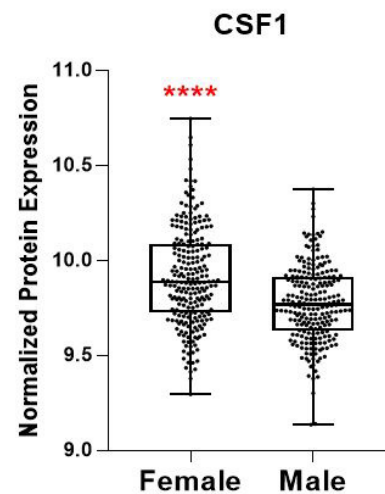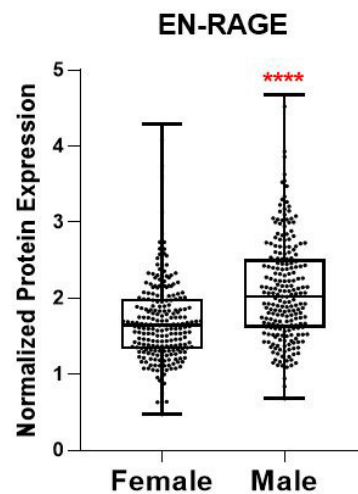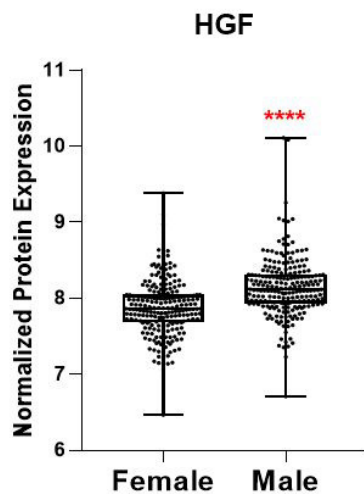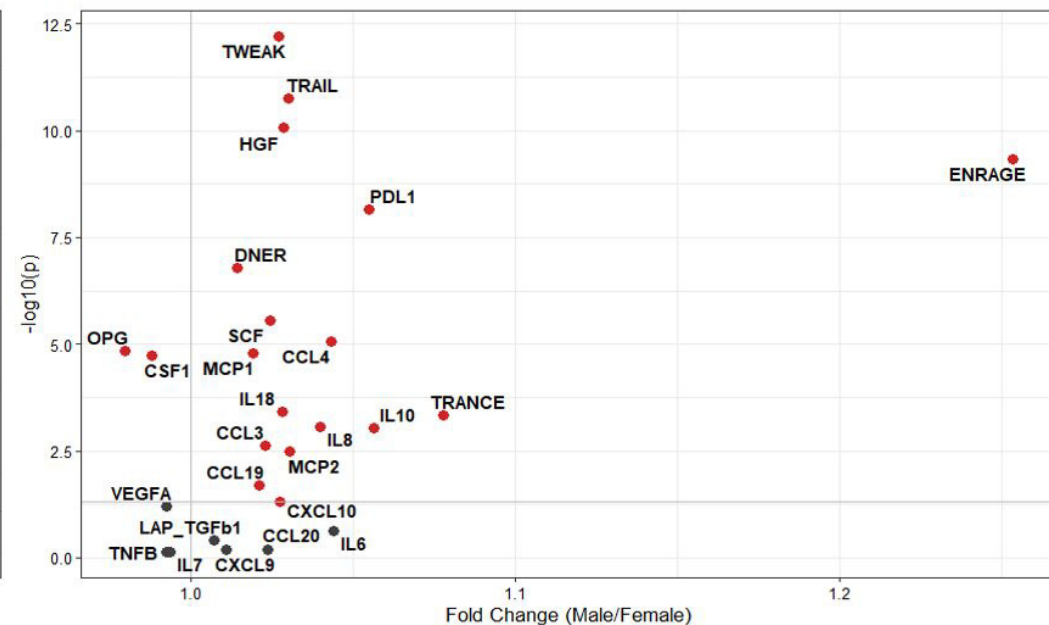
Heat-Inactivated SARS-CoV-2 Stimulation
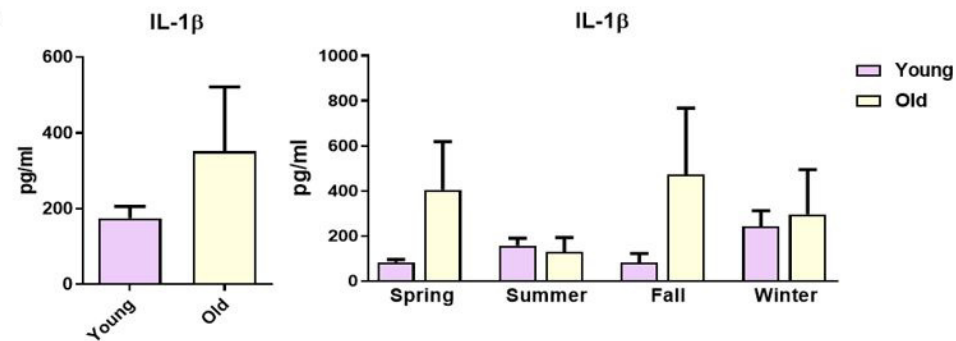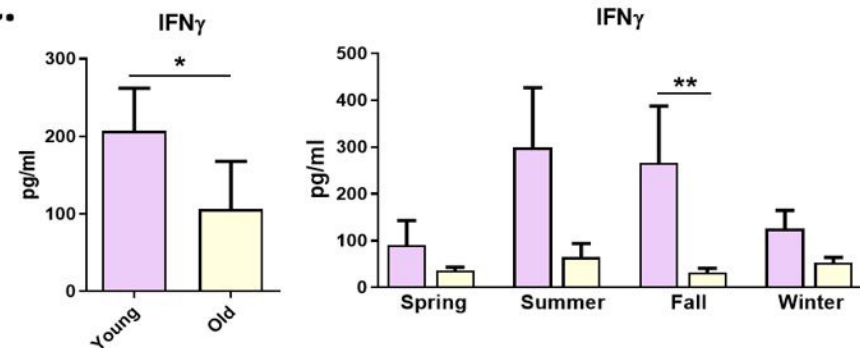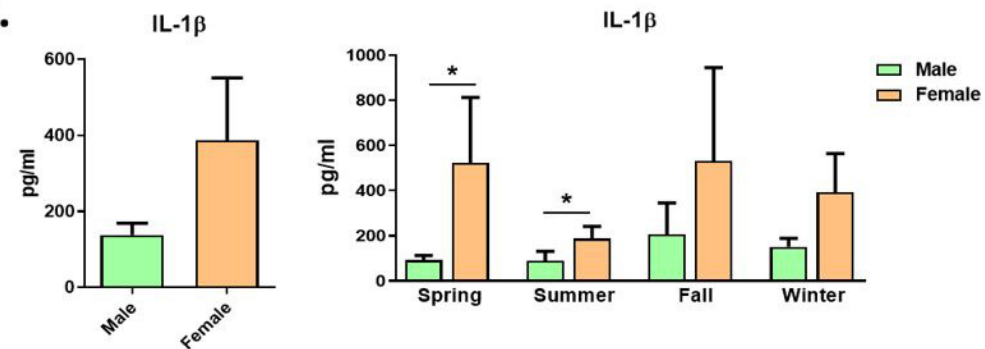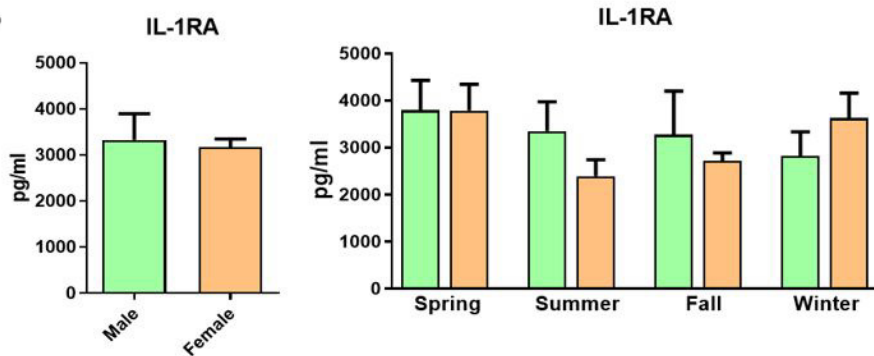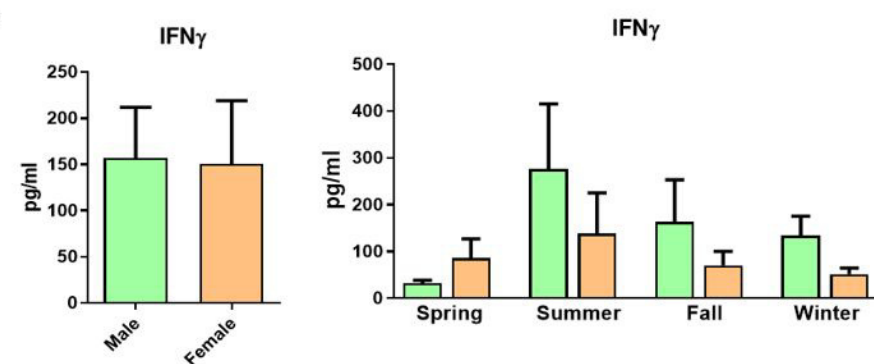
CELL TYPE	HIGH IN SEVERE COVID-19	HEALTHY COHORTS	
		INCREASES WITH AGE	HIGHER IN MALES
Memory Tregs	✓	✓	
PROTEIN			
IL6	✓	✓	
IL8	✓	✓	✓
IL10	✓		✓
IL18	✓	✓	✓
MCP1	✓	✓	✓
MCP2	✓		✓
CCL3	✓		✓
CCL4	✓		✓
CCL19	✓		✓
CXCL10	✓		✓
PDL1	✓		✓
HGF	✓	✓	✓
OPG	✓	✓	
ENRAGE	✓		✓

CELL TYPE	LOW IN SEVERE COVID-19	HEALTHY COHORTS	
		DECREASES WITH AGE	HIGHER IN FEMALES
CD56high NK cells	✓	✓	✓
T cells	✓	✓	✓
Naive CD4 T Cells	✓	✓	✓
Naive CD8 T Cells	✓	✓	✓
Tregs	✓	✓	
Naive Tregs	✓	✓	
B Cells	✓	✓	
Naive B Cells	✓	✓	
PROTEIN			
TRANCE	✓	✓	