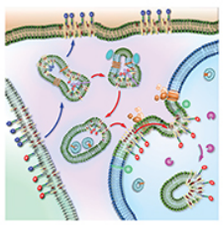
Autophagy



Autophagy

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BRIEF REPORT

The polymorphism L412F in *TLR3* inhibits autophagy and is a marker of severe COVID-19 in males

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ABSTRACT

The polymorphism L412F in TLR3 has been associated with several infectious diseases. However, the mechanism underlying this association is still unexplored. Here, we show that the L412F polymorphism in TLR3 is a marker of severity in COVID-19. This association increases in the sub-cohort of males. Impaired macroautophagy/autophagy and reduced TNF/TNFα production was demonstrated in HEK293 cells transfected with $TLR3^{L412F}$-encoding plasmid and stimulated with specific agonist poly(I:C). A statistically significant reduced survival at 28 days was shown in L412F COVID-19 patients treated with the autophagy-inhibitor hydroxychloroquine ($p = 0.038$). An increased frequency of autoimmune disorders such as co-morbidity was found in L412F COVID-19 males with specific class II HLA haplotypes prone to autoantigen presentation. Our analyses indicate that L412F polymorphism makes males at risk of severe COVID-19 and provides a rationale for reinterpreting clinical trials considering autophagy pathways.

Abbreviations: AP: autophagosome; AUC: area under the curve; BafA1: bafilomycin A1; COVID-19: coronavirus disease-2019; HCQ: hydroxychloroquine; RAP: rapamycin; ROC: receiver operating characteristic; SARS-CoV-2: severe acute respiratory syndrome coronavirus 2; TLR: toll like receptor; TNF/TNF-α: tumor necrosis factor

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Introduction

In December 2019, a new virus was isolated in Wuhan, China, which was called Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2). SARS-CoV-2 is an enveloped positive-sense RNA virus that caused a new pandemic, which WHO named COVID-19 (coronavirus disease-2019).

To date, many characteristics of SARS-CoV-2 are still unclear and, although its ability to be transmitted from one person to another has been ascertained, uncertainties remain about the exact modes of transmission and pathogenicity. In addition, a high variability of symptoms in infected patients and between different populations has been reported; one of the possible explanations of such variability is the genetic background of the host that may affect immune responses to the virus. Among host genetic factors that might impact on symptoms severity there are genes involved in virus entry and mediators of innate immunity [1,2]. TLRs (toll like receptors) are a class of proteins that play a key role in host innate immunity, causing the production of pro-inflammatory cytokines (TNF, IL1, and IL6) and type I and II Interferons, that are responsible for innate antiviral responses. Among TLR genes, *TLR3* encodes an interferon-inducing dsRNA sensor,

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Supplemental data for this article can be accessed here.

whose activation is involved in protection against different RNA viruses [2–4]. Upon viral infection, TLR3 signaling leads to the activation of two factors, NFKB and IRF3 (interferon regulatory factor 3), which play an essential role in the immune response. This results in the production of various cytokines, including TNF (tumor necrosis factor), activating immune responses. However, increased inflammatory responses can make the patient more susceptible to pneumonia and autoimmune diseases. Accordingly, a protective effect against fatal pneumonia has been reported in the absence of TLR3 [5–7]. Among TLR3 variants, the functional L412F polymorphism (rs3775291; c.1234 C > T) is known to decrease TLR3 expression on the cell surface [8]. This polymorphism also leads to poor recognition of SARS-CoV-2 dsRNA, during replication, compared to its wild-type (WT) counterpart [9] and has been recently associated with SARS-CoV-2 susceptibility and mortality [10].

There is evidence that TLR3, as with other TLRs, acts through autophagy in determining susceptibility to infections [11]. The autophagic pathway is essential during infection and for molecular processes such as cell maintenance and homeostasis [12,13]. Indeed, autophagy is one of the major cell defense mechanisms against pathogens [14]. A role for autophagy is reported in different studies on other coronaviruses such as the mouse hepatitis virus/MHV and the transmissible gastroenteritis virus/TGEV [15,16]. A role in SARS-CoV-2 infection has also been described [17–19]. In particular, SARS-CoV-2 can inhibit autophagy resulting in accumulation of autophagosomes and inhibition of viral clearance that, together with immune dysfunction and the activation of numerous inflammatory cytokines, leads to a more severe form of COVID-19 [20–22].

To shed light on the mechanisms underlying the diverse susceptibility to COVID-19, we performed a nested-control study within our GEN-COVID cohort, confirming the role of L412F polymorphism in the *TLR3* gene in susceptibility to SARS-CoV-2 and further defining the potential mechanisms by which this effect is exerted.

Results and discussion

Comparing the extreme phenotypes of SARS-CoV-2 infection, severe COVID-19 patients (cases) versus SARS-CoV-2 PCR-positive oligo-asymptomatic subjects (controls), and using LASSO Logistic regression on common bi-allelic polymorphisms from whole-exome sequencing, we identified the L412F polymorphism (rs3775291; c.1234 C > T) in *TLR3* as a severity marker (Figure 1A). The grid search curve of the cross-validation score (Figure 1B) shows a maximum of the regularization parameter in 10. With this calibration setting, the 10-fold cross-validation provides good performances in terms of accuracy (73%), precision (74%), sensitivity (73%), and specificity (73%) as shown in Figure 1C. The confusion matrix is reported in Figure 1D, whereas the receiver operating characteristic (ROC) curve (Figure 1E) provides an area under the curve (AUC) score of 80%.

The L412F polymorphism has an overall allele frequency of about 20%, ranging from 30% in European to 0.88% in African (mainly sub Saharan) populations [8]. It is intriguing that a relatively COVID-19-free population such as sub Saharan has a very low frequency (0.88%) of this polymorphism and that Asian (26.97%) and European (30.01%) have a much higher frequency. The variant protein with phenylalanine is under-represented on the cell surface, it is not efficiently secreted into the culture medium when expressed as the soluble ectodomain, and it has reduced capability to activate the expression of TLR3-dependent reporter constructs [8]. In order to confirm the role of the polymorphism, we compared individuals showing severe COVID-19 (cases) and those with no sign of the disease (controls). We subdivided patients into two categories, those having the polymorphism in heterozygous or homozygous state and those homozygous for the WT allele. We found that the prevalence of L412F polymorphism is significantly higher in cases compared to controls (p-value 2.8×10^{-2}) (Table 1). The global allele frequency of L412F in our cohort (cases and controls) is 29.38%, comparable to the allele frequency of 29.79% reported in the European (non-Finnish) population in the gnomAD database (https://gnomad.broadinstitute.org/). The identified frequencies were in Hardy-Weinberg equilibrium.

Sex-related differences of TLRs activation following stimulation by viral nucleic acid may be involved in the sex-related variability in response to viral infections [23]. Several rare TLR3 loss of function mutations are known to be linked both to influenza and SARS-CoV-2 virus as well as hyperfunctioning mutations [24,25]. In agreement with these data, when we stratified by gender, the statistically significant difference increased in the sub-cohort of males giving an Odds Ratio of 1.94 (95% confidence interval, 1.23 to 3.06; p = $3.8\text{x}10^{-3}$), whereas it was lost in the sub-cohort of females (p-value 5.8×10^{-1}) (Tables 1, 2, 3).

We then investigated the prevalence of patients carrying L412F in heterozygous or homozygous states in all 4 categories of COVID-19 clinical severity, considering only male subjects regardless of age (n = 665). We found that the prevalence of carriers directly increased with the severity of COVID-19, from a clinical condition not-requiring hospitalization to intratracheal intubation (Figure 1F)

The L412F substitution in TLR3 falls in the ectodomain, in the 14 leucine-rich repeats/LRR domain, a motif of 22 amino acids in length that folds into a horseshoe shape [26]. Proteins containing leucine-rich repeats are involved in a variety of biological processes, including signal transduction, cell adhesion, DNA repair, recombination, transcription, RNA processing, disease resistance, apoptosis, and the immune response [27]. The L412F substitution is expected to have a limited structural impact with minimal rearrangement of near hydrophobic amino acids such as tryptophan 386 (Figure 2). However, the absence of one of the leucines probably determines a different rearrangement of the motif and consequently of the near glycosylation site Asn414, having an impact on protein-protein interaction and in signal transduction process [28].

Germline knockout of *TLR3* inhibits autophagy and upregulation of TLR3 promotes damage after myocardial infarction mainly because of autophagy rather than inflammatory activation [29]. Interestingly, we could notice a statistically significant (p = $3.8\text{x}10^{-2}$) reduced survival at 28 days in TLR3_L412F COVID-19 patients treated with

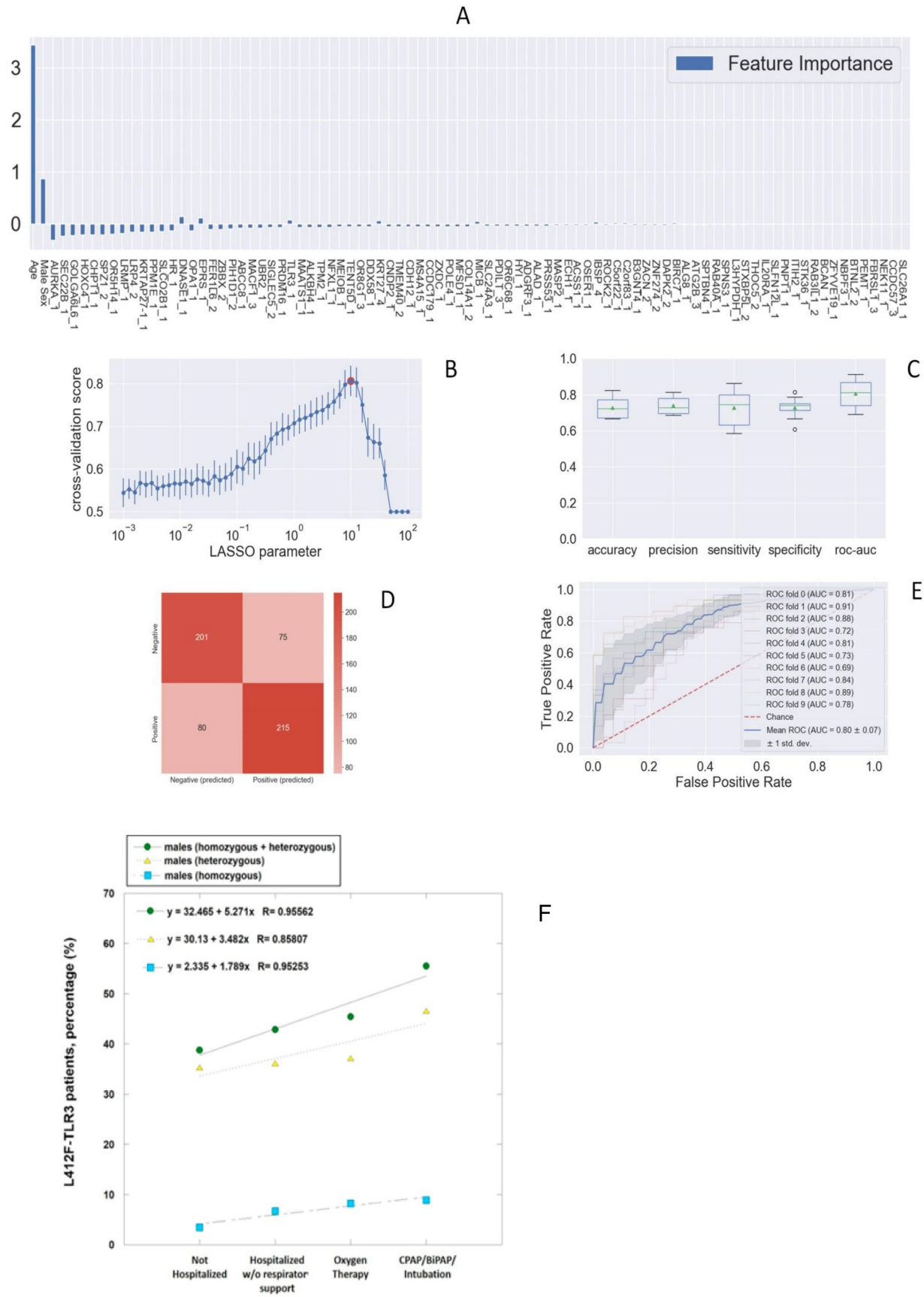

Figure 1. The histogram of the LASSO logistic regression weights represents the importance of each feature for the classification task, (A) The positive weights reflect a susceptible behavior of the features to the target COVID-19 disease, whereas the negative weights a protective action. (B) Cross-validation ROC-AUC score for the grid of LASSO regularization parameters; the error bar is given by the standard deviation of the score within the 10 folds; the optimal regularization parameter is chosen by selecting the one with highest cross-validation score (red point). (C) Boxplot of accuracy, precision, sensitivity, specificity, and ROC-AUC score for the 10-fold of the cross-validation. The box extends from the Q1 to Q3 quartile, with a line at the median (Q2) and a triangle for the average. (D) Confusion matrix for the aggregation of the logistic regression predictions in the 10 folds of the cross-validation. (E) ROC curve for the 10 folds of the cross-validation. (F) Distribution of carriers of the polymorphism L412F in homozygous or heterozygous states stratified by clinical category.

Table 1. L412F and COVID-19 outcome (both sexes).

	Cases	Controls	Marginal Row Totals
L412F	186 (55.0%)	139 (46.3%)	325 (50.9%)
Wild-Type	152 (45.0%)	161 (53.7%)	313 (49.05%)
Marginal Column Totals	338 (52.97%)	300 (47.02%)	638 (Grand Total)

p-value (cases vs controls) = $2.8x10^{-2}$

Table 2. L412F and COVID-19 outcome (males only).

	Cases	Controls	Marginal Row Totals
L412F	131 (55.3%)	45 (38.8%)	176 (49.8%)
Wild-Type	106 (44.7%)	71 (61.2%)	177 (50.1%)
Marginal Column Totals	237 (67.13%)	116 (32.86%)	353 (Grand Total)

p-value (cases vs controls) = $3.6x10^{-3}$

Table 3. L412F and COVID-19 outcome (females only).

	Cases	Controls	Marginal Row Totals
L412F	55 (54.5%)	94 (51.1%)	149 (5.,3%)
Wild-Type	46 (45.5%)	90 (48.9%)	136 (47.7%)
Marginal Column Totals	101 (35.43%)	184 (64,56%)	285 (Grand Total)

p-value (cases vs controls) = $5.8x10^{-1}$

hydroxychloroquine (HCQ) (Figure 3A). As this drug is a well-established inhibitor of autophagy, we reasoned that alterations of this important biological process might have a role in the increased severity of the clinical phenotypes of SARS-CoV-2 infection in patients with the $TLR3^{L412F}$ polymorphism. Notably, beside being entirely ineffective at changing the clinical evolution of COVID-19, which led to retraction of the paper reporting the clinical trial [30], HCQ may have been responsible for reportedly increased fatality rates among patients treated with this drug [31,32]. Poly(I:C) stimulation of the TLR3 receptor has already been shown to stimulate autophagy [29]. Therefore, we decided to compare the efficacy of transfected WT and L412F-mutated receptors in inducing autophagy upon poly(I:C) treatment. To monitor autophagy, we used indirect immunofluorescence microscopy to score the formation of punctate intracellular vacuoles stained for LC3B. Moreover, to better appreciate autophagosomal formation, we also used bafilomycin A_1 (Baf A1), an inhibitor of lysosomal acidification, to prevent lysosomal degradation of autophagosome-associated LC3B [33]. Baf A1 allows, in fact, the detection of each autophagosome formed in the time-lapse between addition of the drug to cells and harvesting [33]. Importantly, to avoid potentially confounding effects of the endogenous TLR3 receptor in transfected cells, we used *TLR3* knockout HEK cells. In these cells, when transfected with a plasmid encoding WT TLR3 (TLR3_WT), we observed a progressively increasing number of autophagosomes (APs) when stimulated with poly(I:C) for different time points in the presence of BAF A1 (compared to BAF A1 alone) (Figure 3B-C), indicating a stimulation of the synthesis of these vesicles and a positive autophagic flux. Conversely, in HEK cells transfected with TLR3_L412F, the number of AP was reduced by poly(I:C) stimulation in the presence of BAF A1 (Figure 3B-C), demonstrating a block in AP synthesis and a reduced autophagic flux. Interestingly, in the absence of BAF A1, AP numbers did not increase upon poly(I:C) stimulation, suggesting that, in HEK cells, fast degradation of AP may compensate for a small increase in the synthesis. Indeed, also rapamycin (RAP), a strong stimulus for autophagy [34], induced only a small increase of AP in the absence of BAF A1 in these cells upon transfection of both TLR3_WT and TLR3_L412F, supporting slow rates of AP synthesis in these cells upon stimulation with different stimuli. The fusion process of autophagosomes with hydrolase-containing lysosomes represents the final step in the degradation process along the autophagic route and its evaluation provides important information for flux analysis [33]. Therefore, as a further confirmation of a reduced autophagic flux in HEK cells expressing the $TLR3^{L412F}$ mutant as compared to TLR3_WT-transfected cells, we decided to score fusion of autophagosomes to lysosomes in these cells upon poly(I:C) stimulation, by measuring colocalization rate of LC3B and LAMP-1, through analysis with the Volocity software of immunocytochemistry experiments (**Fig. S1**).

During SARS-CoV-2 infection, adipocytes produce pro-inflammatory cytokines like TNF, IL6 and IL1B/IL-1β which recruit immune cells to the site of infection [35]. Autophagy is stimulated and regulated by these pro-inflammatory cytokines [35]. TNF is a potent immunomodulator and proinflammatory cytokine that has been implicated in the pathogenesis of

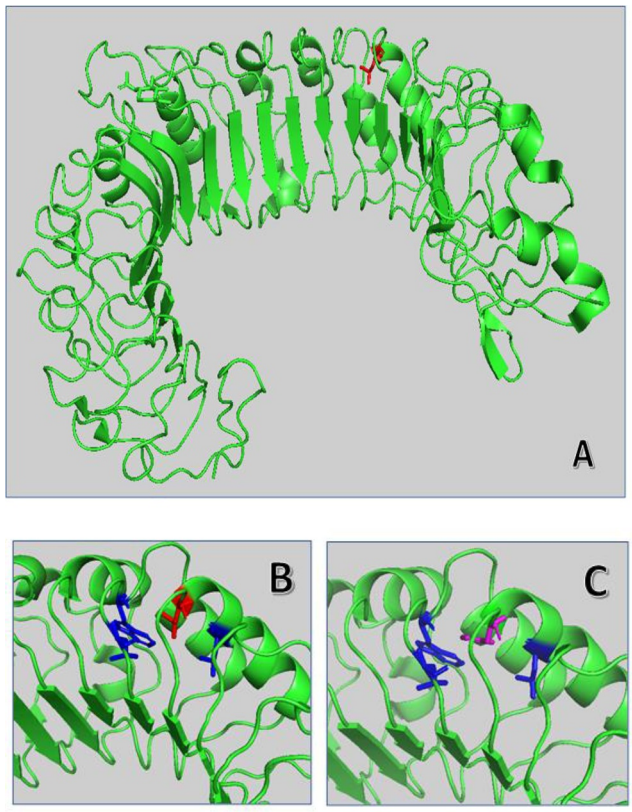

Figure 2. Superposition of wild-type and mutated TLR3 protein. (A) TRL3 human protein tridimensional structure of 2Z7X crystal structure. In green cartoon representation of TLR3 protein. (B) and (C) Zoom of the mutated region with Leu412 in red sticks and Phe412 in magenta. The hydrophobic core of Leu377, Leu389, and Trp386 is in blue sticks.

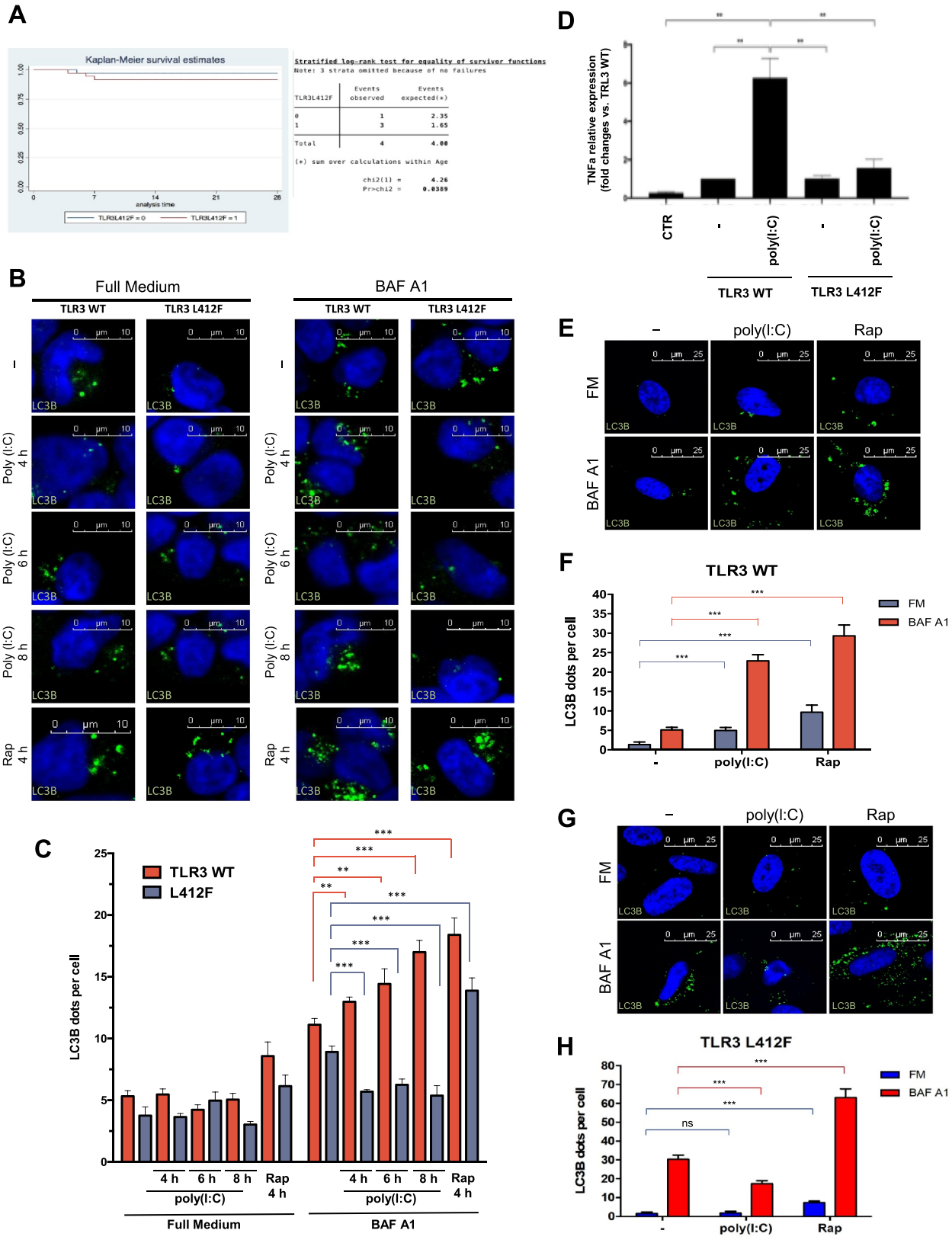

Figure 3. Analysis of autophagy in TLR3_L412F-expressing cells. (A) 28-day survival study of TLR3- L412F carriers vs not-carriers in the group treated with hydroxychloroquine. N = 156, with 73 carriers of TLR3- L412F. Three carriers and 1 not-carrier died in the first 28 days of treatment. (B) Analysis of autophagy in HEK-KO cells expressing wild type or L412F mutant proteins. HEK-KO cells were transfected for 24 h with plasmids encoding TLR3_WT and TLR3_L412F. Cells were next incubated in full medium (FM) or FM + 400 nM bafilomycin A_1 (BAF A1) for 3 h and stimulated for increasing times with 50 μg/ml poly(I:C), as indicated. Cells were next fixed, permeabilized with 100 μg/ml digitonin and stained with anti-LC3B antibodies and revealed with Alexa Fluor 488-conjugated secondary antibodies. Nuclei were stained with DAPI. Where indicated, RAP (500 nM, for 2 h) was used as positive control for induction of autophagy. (C) Same as in B, but the amount of autophagosomes (scored as LC3B-positive dots) per cell was quantified by Volocity software. Measures were obtained by analyzing at least 400 cells/sample from 3 different experiments (n = 3). (D) Analysis of *TNF* mRNA expression in HEK-KO cells expressing wild type or L412F mutant proteins. HEK-KO cells were transfected for 24 h with plasmids encoding empty vector (CTR), TLR3_WT and TLR3_L412F and next stimulated for increasing times with 50 μg/ml poly(I:C), where indicated. TNF

levels were evaluated by Real Time PCR. The gene expression levels were evaluated by the fold change versus TLR2 WT sample using the equation 2^{-DDCt}. Data are presented as the mean ± SEM. Data significance was analyzed using One-way ANOVA test with Holm-Sidak's correction. Asterisks were attributed for the following significance values: P > 0.05 (ns), P < 0.05 (*) and P < 0.01 (**). (E) Normal human fibroblasts (NDHF) from subjects expressing the TLR3_WT receptor were stimulated with 50 µg/ml poly(I:C) or RAP (1 µ M) for 4 h, in full medium alone or containing 400 nM bafilomycin A_1 for 3 h. Cells were next fixed, permeabilized with 100 µg/ml digitonin and stained with anti-LC3B antibodies and revealed with Alexa Fluor 488-conjugated secondary antibodies. Nuclei were stained with DAPI. (F) Same as in E, but the number of autophagosomes (scored as LC3B-positive dots) per cell was evaluated for each sample by Volocity software. (G and H) same as in E-F, but fibroblasts are homozygous for the TLR3_L412F receptor. Statistical analysis was performed using Student's t test. Means § SEM for each value are shown in the graphs. ns = not significant; ** = p < 0.01; *** = p < 0.001. < 0.001.

autoimmune and infectious diseases. It is produced by activated monocytes and macrophages, as well as by many other cell types, including lymphocytes, as a transmembrane protein. Through cell modulation, TNF can activate both cell death and survival mechanisms. TNF induces autophagy through a feedback mechanism, causing further recruitment and activation of lymphocytes and contributing to the excess inflammation typical of SARS-CoV-2 infection [36]. As chloroquine, a powerful inhibitor of autophagy, inhibits production of different cytokines, among which TNF [37], we next decided to test if the inhibitory effect of the L412F mutation on autophagy was also able to mimic the effect of the pharmacological inhibitor of this process in HEK cells. Indeed, while poly(I:C) readily stimulated TNF expression in HEK cells transfected with the TLR3_WT receptor, this effect was completely abolished in TLR3_L412F-transfected cells (Figure 3D).

In order to validate data obtained on transfected HEK cells, we next isolated and cultured skin fibroblasts from healthy donors with different genotypes relative to the TLR3 locus: wild-type (WT/WT) and L412F (L412F/L412F) homozygous. In these primary fibroblasts, immunofluorescence analysis revealed that the number of LC3B-positive vesicles increased upon poly(I:C) stimulation both in the absence and in the presence of BAF A1 (Figure 3E-F), showing an overall positive autophagic flux in WT cells, while the flux resulted significantly reduced in L412F/L412F fibroblasts (Figure 3G-H). As a control, RAP stimulation of both WT/WT and L412F/L412F showed a positive autophagic flux (Figure 3(E,F,G,H) confirming that the mutation specifically affected TLR3-dependent autophagy and not the general autophagic process. Also in these cells, we confirmed a reduced autophagic flux in TLR3_L412F-expressing fibroblasts as compared to TLR3_WT-expressing cells, upon stimulation with poly(I:C), by measurement of the LC3B-LAMP1 colocalization rate (**Fig. S2**).

Overall, our results therefore suggest that the outcome of clinical trials with HCQ should be reinterpreted in the light of $TLR3^{L412F}$ polymorphism status. Negative effects of the drug in L412F bearing subjects may have masked a possible positive outcome in L412F-free subjects. Importantly, they also support a positive role of autophagy in the anti-viral response of the organism to SARS-CoV-2, as suggested by a recent report by Hayn and colleagues [38], demonstrating that at least 3 viral proteins are able to specifically block autophagic turnover.

TLR3 variant L412F has been associated with a wide range of autoimmune diseases including Addison disease and hypothyroidism [39]. *TLR3* rare variants resulting in partial loss of function and occurring together with the common variant L412F, or with another rare variant, have been identified in Addison disease [40]. Persistent viral infections in a background of defective innate immunity lead to overexpression of HLA allotypes prone to present autoantigen. Defects of autophagy have been observed in many infectious and autoimmune diseases. Alteration of autophagic processes causes the onset of autoimmunity due to increased survival and reduced apoptosis of self-reactive lymphocytes [41–43]. HLA has been shown to be implicated in disease severity and clinical outcome of patients with COVID-19 [44]. Accordingly, an increased frequency of autoimmune disorders as co-morbidity was found in our cohort in L412F COVID-19 patients with specific HLA class II haplotypes prone to autoantigen presentation. In particular, we analyzed the DR3-DQ2 haplotype which predisposes to different types of autoimmune diseases [45,46]. The frequency of autoimmune disorders is indeed significantly increased in male patients with HLA DR3/DQ2 haplotype and L412F, especially diabetes (25%) (Table 4 **and** Table 5). These results suggest that the combination of L412F in *TLR3* and a specific class II HLA haplotype puts male patients at risk of post-COVID autoimmune exacerbation emphasizing the need for appropriate follow-up.

No association was found between AIRE loss of function variants and COVID-19 outcome, as outlined by the absence of the gene in Figure 1.

In conclusion, we have identified the second protein-encoding polymorphism that modulates COVID-19 outcome. These results indicate that L412F polymorphism in the *TLR3* gene makes males, in whom after puberty testosterone lowers TLR3 expression, at risk of severe COVID-19 in a context of a polygenic model. Moreover, based on impairment of autophagy, these data provide a rationale for reinterpreting clinical trials with HCQ stratifying patients by L412F. Finally, the combination of L412F in *TLR3* and specific HLA class II haplotypes may put male patients at risk of post-acute sequelae of SARS-CoV-2 infection pointing to the need for an appropriate follow-up. Our experiments suggest an important role of autophagy downstream of the TLR3 receptor, possibly affecting TNF production and susceptibility to infections, including SARS-CoV-2, pinpointing to IFNs treatment (especially IFN γ) avoiding hydroxiclorochine.

Table 4. Association between DR3-DQ2 + L412F haplotype and autoimmune disorders in male patients.

	With autoimmune disease	W/O autoimmune disease	Marginal Row Totals
DR3-DQ2 + L412F	12 (3.59%)	12 (3.59%)	24 (7.18%)
Other	64 (19.16%)	246 (73.65%)	310 (92.81%)
Marginal Column Totals	76 (22.75%)	258 (77.24%)	334 (Grand Total)

p-value (cases vs controls) = 0.000951

Table 5. Male patients with L412F and HLA DR3/DQ2 haplotype.

PatientID	Age	Ethnicity (white = 1, hispanic = 4)	Clinical Category	GT TLR3 (L412F)	Clinical known comorbidities
AR-COV-25	69	1	4	0/1	Hypertension, Kidney failure, COLD
BS-COV-102	59	1	3	0/1	Diabetes Mellitus, diabetic neuropathy with transmetarsal amputation, bilateral fachiectomy, left hernioplasty, diabetic retinopathy
BS-COV-58	47	1	3	0/1	Hypertension
BS-COV-65	72	1	2	0/1	Rheumatoid Arthritis
BS-COV-70	65	1	3	0/1	Autoimmune hepatitis, autoimmune polyghiandol syndrome, previous autoimmune thyroid
CR-COV-17	61	1	2	0/1	Inflammatory disease, Hypertension
CR-COV-4	66	1	3	0/1	None
LS-COV-10	37	1	1	0/1	DLB-CL EBV+, Immunodeficiency, Bipolar Disorder
LS-COV-19	58	4	2	0/1	Diabetes Mellitus
LS-COV-8	48	1	4	0/1	Hypertension
MORE-COV-16	65	1	3	0/1	Hypertension, Asthma, Dyslipidemia
MORE-COV-7	72	1	4	0/1	Hypertension
PG-COV-15	87	1	2	0/1	Congestive heart failure, Dyslipidemia, Stroke
PG-COV-23	68	1	1	0/1	N/A
PV-COV-02	65	1	2	0/1	Rheumatoid Arthritis
PV-COV-23	72	1	3	0/1	Hypertension, Diabetes Mellitus
PV-COV-24	79	1	2	0/1	Atrial Fibrillation, Diabetes Mellitus, Kidney failure
PV-COV-39	33	4	4	0/1	Diabetes Mellitus, Obesity
PV-COV-73	54	1	3	0/1	None
PV-COV-97	66	1	4	0/1	Congestive Heart Failure, Hypertension, Diabetes Mellitus
RUF-COV-1	52	1	0	0/1	None
SPC-COV-14	56	1	3	0/1	Asthma, HCV
TV-COV-81	44	1	1	0/1	None
TV-COV-96	51	1	2	0/1	Hypertension

Materials and methods

Patients

We performed a nested case-control study (NCC). We used a cohort of 1319 subjects (cases and controls) from the Italian GEN-COVID Multicenter study, infected with SARS-CoV-2 diagnosed by RT-PCR on nasopharyngeal swab [47]. Cases were defined as patients needing endotracheal intubation or CPAP/biPAP ventilation. Controls were oligo-asymptomatic subjects not requiring hospitalization.

Ethics approval

The GEN-COVID study was approved by the University Hospital of Siena Ethical Review Board (Protocol n. 16,929, dated 16 March 2020).

LASSO logistic regression

We adopted the $\lambda \sum_{k=1}^{p} |\beta_k|$Least Absolute Shrinkage and Selection Operator (LASSO) logistic regression model for the classification of severe COVID-19 patients (cases) versus SARS-CoV-2 PCR-positive oligo-asymptomatic subjects (controls), able to enforce both the sparsity and the interpretability of the results. By denoting with β_k the coefficients of the logistic regression and by lambda (λ) the strength of the regularization, the LASSO regularization [48] term of the loss, has the effect of shrinking the estimated coefficients to 0. In this way, the weights of the logistic regression algorithm can be interpreted as the feature importances of the subset of the most relevant features for the task [49]. The input features are the common bi-allelic polymorphisms from whole-exome sequencing as well as gender, and the age, the latter as a continuous variable normalized between 0 and 1. Common bi-allelic polymorphisms are defined as combinations of two polymorphisms, each with minor allele frequency above 1%, with frequency above 5% in the cohort.

The fundamental hyper-parameter of the logistic regression algorithm is the strength of the LASSO term, which is tuned with a grid search method on the average area under the ROC curve for the 10-fold cross-validation. The regularization hyperparameter varies in the range $[10^{-3}, 10^{2}]$ with 50 equally spaced values in the logarithmic scale. The optimal regularization parameter is chosen by selecting the parameter with the highest cross-validation score. During the fitting procedure, the class slight unbalancing is tackled by penalizing the misclassification of the minority class with a multiplicative factor inversely proportional to the class frequencies. The data preprocessing was coded in Python, whereas for the logistic regression model the scikit-learn module with the liblinear coordinate descent optimization algorithm was used. Performances of the model were evaluated using the cross-validation confusion matrix as well as by computing precision, sensitivity, specificity, and the ROC curve.

Cell culture and transfection

HEK-Dual™ Null (NF/IL8) cells (Invivogen, hkd-nullni) cells were cultured in Dulbecco modified Eagle medium (DMEM; Euroclone, ECB7501L) supplemented with 10% fetal bovine serum (FBS; Euroclone, ECS0180L), 2 mM L-glutamine (Carlo Erba, ABP379-100) and 100 units/ml penicillin-streptomycin (Life Technologies, 15,140,148) at 37°C in an atmosphere of 5% CO_2:air. Transfections were performed with 1 μg DNA plasmid using lipofectamine LTX (Life Technologies, 15,338,500). The cells were seeded to be 70% to 80% confluent, then DNA was diluted in DMEM with 10 mM HEPES, pH 7.2. Lipofectamine LTX was next added

to the complex (5 µl) to allow creation of complexes (30 min at RT). Ultimately, DNA-lipid complexes were added to cells. Bafilomycin A_1 was from Santa Cruz Biotechnology (sc-201,550). Human primary fibroblasts were obtained from the Genetic Biobank of Siena (http://biobanknetwork.telethon.it/; cell line number: Rett 2250, 2980/18, 1031/15, Rett 1200). Fibroblasts were cultured in Dulbecco Modified Eagle medium supplemented with 10% FBS, 2% L-glutamine and 1% penicillin-streptomycin, according to standard protocols, and routinely passed 1:2 with trypsin-EDTA (0.05%) solution (Irvine Scientific, 9342).

Immunofluorescence (IF)

Cells were washed with phosphate-buffered saline (PBS; Oxoid, BR0014G), then fixed with 4% paraformaldehyde in PBS for 20 min, washed with PBS and permeabilized with digitonin solution (Life Technologies, BN2006) for 20 min. Then, the cells were washed three times in PBS. Permeabilized cells were incubated with anti-LC3B (MBL, M152–3) and/or anti-LAMP1 (Cell Signaling Technology, 9091) primary antibodies for 1 h, washed three times with PBS, and then incubated with anti-mouse Alexa Fluor 488-conjugated (Life Technologies, A21202) and/or Alexa Fluor 647 (Life Technologies, A21245) secondary antibodies; subsequently cells were washed three times with PBS. Nuclei were stained with a solution of 6 µM of 4′,6-diamidino-2-phenylindole (DAPI; Sigma Aldrich, D9542) in PBS for 10 min. Coverslips were mounted in a fluorescence mounting medium (Dako, S3023). Samples were visualized on a TSC SP5 confocal microscope (Leica Microsystems, 5,100,000,750) installed on an inverted LEICA DMI 6000CS (Leica Microsystems, 10,741,320) microscope and equipped with an oil immersion PlanApo 63 × 1.4 NA objective. Images were acquired using the LAS AF acquisition software (Leica Microsystems, 10,210). Poly(I:C) was from InvivoGen (31,852–29-6).

Dot count and statistical analysis for autophagy

For the LC3B-positive dot counts, we performed intensitometric analysis of fluorescence using the Quantitation Module of Volocity software (PerkinElmer Life Science). LC3B-LAMP 1 colocalization rate was measured by the Quantification tool of LAS AF software (Leica Microsystems). Dot counts and colocalization rate were subjected to statistical analysis. Measures were obtained by analyzing at least 400 cells/sample (dot counts) or 250 cells/sample (colocalization rate) from 3 different experiments. Significance (P value) was assessed by Student's t test, using GraphPad Prism6 software. Asterisks were attributed for the following significance values: $P > 0.05$ (ns), $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***).

Real time qPCR analysis of TNF expression

Total RNA was isolated using the RNAeasy Mini Kit (Qiagen, NC9677589) according to the manufacturer's instructions. cDNA synthesis was performed using the Maxima First Strand cDNA Synthesis Kit (Life Technologies, EP0751). Neosynthetized cDNA was used to perform Real Time PCR using the PowerUp Sybr Green (Life Technologies, A25779). The following primers were used: *TNF* Fw CTATCTGGGA GGGGTCTTCC; *TNF* Rw GGTTGAGGGTGTCTGAAGGA; *HPRT1* Fw GTCTTGCTCGAGATGTGATG and *HPRT1* Rw GTAATCCAGCAGGTCAGCAA. Target transcripts were analyzed with the QuantStudio 7 System (Applied Biosystems, CA, USA). The comparative threshold cycle (Ct) method was used for quantification analysis. The Ct values of each gene were normalized to the Ct value of HPRT1. The gene expression levels were evaluated by the fold change using the equation 2-ΔΔCt.

HLA *sequencing*

HLA-class I and *II* genes were targeted for DNA sequencing using a biotinylated DNA probe-based capture method [50], with modifications as follows. Genomic DNA (500 ng from each sample) was fragmented enzymatically using the NEBNext Ultra ii FS module (New England Biolabs, E7810S). Individual samples were labeled uniquely using 3 µl of 15 µM custom dual-index adapters (Integrated DNA Technologies, Coralville, IA, USA) and the NEB ligation module. Post ligation cleanup was based on the Kapa Hyper Prep protocol (Kapa Biosystems, Wilmington, MA) and followed by dual size selection. Paired ends of 250 bp each were sequenced using a NovaSeq instrument and SP Reagent Kit (Illumina Inc, San Diego, CA, USA). *HLA* alleles were determined from the sequence data using the consensus from three algorithms: NGSengine 2.10.0 (GenDX, Utrecht, The Netherlands), HLA Twin (Omixon Biocomputing Ltd. Budapest, Hungary) and HLA*LA [51].

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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