Metabolic reprogramming and host-immune response against Crimean-Congo Hemorrhagic Fever Viruses

Short title: Metabolic reprogramming in CCHFV

Authors

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Abstract

The pathogenesis and host-viral interactions of the Crimean–Congo hemorrhagic fever virus (CCHFV) are convoluted and have not been evaluated previously. To understand the host immune responses against CCHFV, we have performed a global transcriptomic analysis of peripheral blood mononuclear cells from a longitudinal cohort of CCHF patients who survived and temporal untargeted proteomics analysis of CCHFV infected cells. Our results indicate that during the acute phase of CCHFV infection, the host's metabolic reprogramming towards central carbon metabolism including glycolysis/gluconeogenesis occurs. This could be regulated by the PI3K/Akt, HIF-1, FoxO, and AMPK signaling pathways and play a central role in viral replication. Moreover, key interferon stimulating genes (ISGs; ISG12, ISG15, ISG20 and MXs; Mx1 and Mx2) are activated during infection, suggesting a role for type I and II interferon-mediated antiviral mechanisms. Targeting type I interferon response through metabolic perturbation could be an attractive therapeutic intervention for CCHFV as well as aid as antiviral strategies.

51 Introduction

52 Crimean–Congo hemorrhagic fever orthonairovirus (CCHFV) is considered to be a major
 53 emerging viral threat causing an increasing number of outbreaks in several parts of the globe.
 54 CCHFV is a negative-sense RNA virus belonging to the *Nairoviridae* virus family, which causes
 55 a mild to severe viral hemorrhagic fever. Crimean–Congo hemorrhagic fever (CCHF) poses a
 56 substantial threat to public health due to its high mortality rate in humans (3–40%), modes of
 57 transmission (tick-to-human/animal, animal-to-human and human-to-human) and geographical
 58 distribution (WHO, 2020). The Ixodid ticks, especially those of the genus, *Hyalomma*, are both a
 59 vector and a reservoir for CCHFV and are highly ubiquitous with its presence in more than 40
 60 countries (ECDC, 2020). CCHF is endemic in almost 30 countries that are widely distributed
 61 throughout sub-Saharan Africa, South-Eastern Europe, the Middle-East, Central Asia down to
 62 Pakistan, and as far as east as the Xinjiang province of Northwest China(Bente, Forrester et al.,
 63 2013, Zivcec, Scholte et al., 2016). In recent years, CCHFV outbreaks have become more
 64 frequent and have spread to new geographical areas; this has been attributed to climate changes
 65 and the spread of infected ticks by birds and the livestock trade. The presence of the CCHFV
 66 vector in Portugal, Spain, Germany, and even Sweden (Grandi, Chitimia-Dobler et al., 2020) and
 67 England (McGinley, Hansford et al., 2020) highlights the need for stricter surveillance due to the
 68 possibility of a future epidemic(Estrada-Peña, Sánchez et al., 2012).

69 Type-I interferon (IFN-I) is one of the first-line of antiviral innate immune defenses against
 70 viruses. Following infection, viral RNA or its replication products can induce secretion of IFN-I
 71 that subsequently stimulates interferon-stimulated genes (ISGs) in a tightly controlled signaling
 72 cascade. Thus, components of the IFN-I signaling pathway are often targeted by viruses to evade
 73 the antiviral immune response. It is known that CCHFV replication is sensitive to IFN-I
 74 (Andersson, Lundkvist et al., 2006). However, the virus is also able to delay the induction of IFN,
 75 and IFN treatment has been shown to be ineffective following establishment of infection,
 76 suggesting that CCHFV has developed mechanisms to block innate immune
 77 responses(Andersson, Karlberg et al., 2008). In a limited number of studies, CCHF patients were
 78 shown to have elevated innate immune response-associated cytokines and chemokines, of which
 79 IL-6 and TNF- α were often associated with mortality (Kaya, Elaldi et al., 2014, Korva, Rus et al.,
 80 2019, Weber & Mirazimi, 2008). CCHF was also observed to be associated with increased
 81 secretion of IFN- α 2 and IFN- γ , irrespective of severity and disease fatality(Korva et al., 2019,
 82 Papa, Bino et al., 2006). The existing knowledge from in vitro studies, autopsies of CCHF
 83 patients and clinical findings suggests a regulatory mechanism of the IFN-response during

84 CCHFV infection and indicates that IFN stimulated genes may offer protection by inhibiting
85 CCHFV. However, it is not known which among the more than 1000 ISGs are responsible for the
86 IFN effect against CCHFV or how the strength of viral anti-IFN mechanisms and cytokine
87 profiles might correlate with the virulence level of particular CCHFV strains.

88 Because of the sporadic nature of CCHF outbreaks in humans in the endemic regions, a lack of
89 infrastructure, and the absence of structured studies, little is known about the pathogenesis and
90 host-virus interactions during the acute phase of CCHF disease and associated sequelae after
91 recovery. Understanding the host response to CCHFV is necessary to design better therapeutic
92 and containment strategies for CCHF. Recently, systems biology studies using -omics approaches
93 on patient material and infected cells have offered a route to elucidate potential mechanisms of
94 the host immune response against invading viruses and disease pathogenesis, as recently reported
95 by us in case of SARS-CoV-2 infection (Appelberg, Gupta et al., 2020).

96 In this study, we have applied transcriptomics and proteomics analysis to understand the host
97 immune and cellular responses against CCHFV. We have used longitudinal blood samples
98 collected during both the acute phase of CCHFV infection and the convalescent-phase (nearly
99 after a year of recovery), as well as an *in vitro* infection model in Huh7 cells,. Our study is the
100 first to provide a comprehensive understanding of the fundamental innate immune responses that
101 modulate successful CCHFV infection.

102 **Results**

103 **Samples and clinical data**

104 In this study, 18 samples were collected during the acute phase of the disease with a median time
105 of 4 days (range 1-6 days) after the onset of symptoms. By using SGS, 33% (6/18) patients were
106 grouped into severity group 1, 61% (11/18) patients into severity group 2 and, 6% (1/18) patients
107 into severity group 3. The median age of the patients was 49 (range: 18–79) years, and 12
108 (66.7%) of the patients were male. A 79-year-old male patient in severity group 3 died on the
109 third day of hospitalization. The case-fatality-rate (CFR) for the cohort was 5.6%. Follow-up
110 samples were collected from 12 individuals after a median duration of 54 weeks (range: 46-57
111 weeks) (Fig. 1a). The CCHF patient characteristics are summarized individually in
112 Supplementary Table S1.

113 **The impact of CCHF severity on peripheral blood transcriptome during acute infection**

We first considered the effect of the SGS disease severity group on the gene expression profile at the mRNA level. The UMAP analysis using all of the genes did not show distinct differences between samples of different severity groups (Figure 1b). The heterogeneous gene-expression profile was further refined by hierarchical clustering analysis of the top 500 high variable genes (Supplementary Figure S1). To specifically investigate the genes that were significantly associated with disease severity, the samples were grouped into either severity group 1 or severity groups 2 and 3. The differential gene expression (DGE) was plotted as an MA -plot, as a measure of the number of genes whose expression was consistently altered between these two groups (Figure 1c). There were 12 genes (ERG, PROM1, HP, HBD, AHSP, CTSG, PPARG, TIMP4, SMIM10, RNASE1, VSIG4, CMBL, MT1G) that were significantly upregulated in patients in severity groups 2 and 3 compared to severity group 1 (Figure 1c). However, no obvious links between these genes were noted.

Cytokine levels in severity group 1 and severity group 2 during acute CCHFV infection

Serum samples collected during the acute phase of the disease from severity group 1 (n=6) and severity group 2 (n=11) were analyzed using Luminex assay to assess the soluble marker. Of the 22 markers used for analysis, only IL-8 and GM-CSF were significantly higher in severity group 2, and a near significant upregulation was also observed with TNF- α and IL-10 compared to severity group 1 (Figure 1d). The levels of all of the rest 18 biomarkers are shown in Supplementary Figure S2. Cumulatively, the transcriptomics and cytokine array data suggest that there were no significant inflammatory differences with severity in patients who survived CCHF infection.

Transcriptomics changes in the acute phase and post-CCHF recovery

Due to the natural heterogeneity in human cohorts, we used longitudinal samples to perform differential expression analyses for each infected patient between the time of infection and approx. 1 year post-recovery (Range: 46 – 57 weeks). There were 12 patients (severity group 1: n=5; severity group 2: n= 7) available for which sampling was done both during acute infection and post-recovery. We observed a clear separation between the acute phase and the recovered phase samples using UMAP clustering (Supplementary Figure S3).

The DGE profile for the acute phase compared to the recovered phase in all patients showed an upregulation of 2891 genes and a downregulation of 2738. The complete profile is given in Supplementary Data File 1. The pathways that are significantly modulated by the upregulated

genes (FDR<0.05) are mainly related to metabolic pathways like glycolysis/gluconeogenesis, N-glycan biosynthesis and oxidative phosphorylation (OXPHOS), and antiviral pathways like the NOD-like receptor signaling pathway (Figure 2b and Supplementary Data File 2). However, the pathways related to the down-regulated genes were mainly related to antiviral defense mechanism-associated pathways including innate immune responses like Th1, Th2, and Th17, cell differentiation, the NF- κ B pathways, chemokine signaling pathway, etc. (Figure 2c). Interestingly, although the HIF-1 signaling pathway was downregulated, the expression of the HIF-1 targeted genes that play a role in glycolysis/gluconeogenesis and coding for the glycolytic enzymes, Enolase 1 (ENO1) and Phosphoglucomutase 2 (PGM2) was significantly upregulated, while hexokinase 1 (HK1) were downregulated (Supplementary Data File 1). However, when we compared the acute phase with the recovered phase in severity group 1, and severity group 2 separately, there was a distinct DEG profile. In severity group 1 the differentially expressed genes were significantly fewer (n=1617, upregulated: 954 and downregulated: 663) compared to those in severity group 2 (n=4256, upregulated: 2182 and downregulated: 2074) (Figure 2d and 2e). This indicates that the disease severity had a significant effect on gene expression profiling during the acute phase. There were 1451 overlapping genes between severity group 1 and severity group 2 that were differentially up-regulated (n=882) and downregulated (n=569). Using gene ontology (GO) analysis after removal of the redundant terms using REVIGO (Supek, Bošnjak et al., 2011), the top two GO terms that were significantly upregulated were the type I interferon signaling pathway (GO:0060337) and the regulation of viral genome replication (GO:0045069) (Figure 2f).

Interferon signaling pathways in CCHFV infection:

We observed significant differences in gene expression relating to interferon signaling in both severity group 1 and severity group 2, and thus we further explored the alteration of gene expression in all the interferon signaling related pathways. To this end, we used supervised analysis with the genes that are part of 13 GO terms and are part of both type-I and type-II interferon signaling pathways (Figure 3a). Of the IFN-regulated genes, IFI27 (ISG12) showed the most robust upregulation (Figure 3b). This was further supported by the RNAscope analysis targeting the IFI27 transcript in the SW13 cell line infected with CCHFV strain IbAr10200 (Figure 3c). Among the pathways, the maximum significantly differentially expressed genes were part of cellular response to ITN- γ (GO:0071346) (n=57) followed by the Type I interferon (IFN-I) signaling pathway (GO:0060337) (n=35) (Figure 3a) and the majority of genes responsible for IFN-I signaling (89%; 31/35) were upregulated in the acute phase (Figure 3d). IFI27 has been previously shown to be upregulated specifically during virus infection and has the ability to

179 restrict several viral infections. Apart from this ISG20, ISG15, Mx1, Mx2 and several IFN- α -
180 inducible proteins (IRFs) showed upregulation in the acute phase.

181 **Quantitative temporal proteomics analysis of CCHFV infection in Huh7 cell lines:**

182 Our transcriptomics analysis of CCHF patient samples revealed the role of interferon signaling
183 pathways in the pathogenesis of CCHFV, with several ISGs being elevated during the acute phase
184 of infection. To understand the global changes in the host interferome (cluster of interferon genes)
185 during CCHFV infection, we infected Huh7 cells with CCHFV and used a time-course
186 experiment for 24 and 48 hpi. We then performed TMT-based mass-spectrometric analysis
187 (Figure 4a). The global proteome analysis revealed changes in several pathways that were related
188 to metabolism and the organismal system (Figure 4b). This was in line with the changes we
189 observed in the patient transcriptomics analysis (Figure 2b and 2c). Overall, 16 pathways were
190 observed both in patients' transcriptomics and *in vitro* infection assays in Huh7 cells
191 (Supplementary Figure S4).

192 To identify changes in the interferon landscape, we used the GO terms for the cellular response to
193 IFN- γ (GO:0071346) and the Type I interferon signaling pathway (GO:0060337) that showed
194 significant changes in expression in patient samples during acute infection. The overall changes in
195 the interferome over time are represented as a heat-map in Figure 5a. The significant changes in
196 the protein levels at different time points are represented as volcano plots (Figure 5b-c). At 24hpi,
197 several ISGs, like Mx1, ISG20 and IFI16, that were upregulated in the acute-phase patient
198 samples were also significantly elevated in infected Huh7 cells (Figure 5b). Mx1 retained a
199 sustained elevation 48 hpi, and ISG15 was also significantly elevated (Figure 5c). The most
200 interesting ISG was Mx2 that showed a suppression at 24hpi and a significant elevation at 48hpi
201 (Figure 5d).

202 We have previously shown the anti-CCHF role of Mx1 and the interplay between ISG15 and
203 CCHFV is well documented by us (Andersson, Bladh et al., 2004) and others (Scholte, Zivcec et
204 al., 2017). We also observed induction of ISG20, a 3'-to-5' exonuclease that showed elevation in
205 both infected patients and infected cell lines. ISG20 has been reported to have anti-viral activity
206 against a number of bunyaviruses (Feng, Wickenhagen et al., 2018). In order to ascertain its effect
207 on CCHFV replication, we used doxycycline inducible stable cell lines that expressed human
208 ISG20 and CAT protein as a control. Following infection with CCHFV at an MOI of 1 for 48 h,
209 there was some degree of inhibition noted in the doxycycline-induced ISG20 cell line ($p=0.26$)
210 and no inhibition was observed in the CAT control cell line (Figure 5e). Though not significant,
211 the results are suggestive of an antiviral property of ISG20 that is dependent on the enzymatic

212 activity and this requires further investigation. Furthermore, there is also a possibility that the
 213 virus has evolved measures to counteract these antiviral responses. During the full course of
 214 infection, there was significant suppression of IRF3, the transcription factor for IFN β production,
 215 indicating viral mediated evasion or delay in activation of the immune response. The time-
 216 dependent stimulation of different ISGs by CCHFV suggests differential regulation of the IFN
 217 pathway and does not rule out an IFN-I independent mechanism of activation of ISGs.

218

219 Discussion

220 Metabolic reprogramming during innate antiviral immune responses changes the subsequent
 221 phases of disease progression and influences the adaptive immune response. In this study, using
 222 the genome-wide transcriptomic analysis of a longitudinal patient cohort, and temporal
 223 proteomics from *in vitro* infection assays in Huh7 cells, we showed that metabolic
 224 reprogramming could facilitate optimal CCHFV replication and the antiviral mechanism of the
 225 host. Our data indicated that during the acute phase of CCHFV infection there is metabolic
 226 reprogramming of host cells towards central carbon metabolism (e.g. glycolysis/gluconeogenesis,
 227 pyruvate metabolism, citrate cycle/TCA cycle, pentose phosphate pathway etc) that could
 228 potentially be regulated by the PI3K/Akt, HIF-1, FoxO, and AMPK signaling pathways and play
 229 a central role in viral replication (Figure 6 and Supplementary Figure S5). Moreover, type I and II
 230 interferon-mediated antiviral mechanisms were also activated with elevated key antiviral ISGs
 231 (ISG12, ISG15, ISG20), and MXs (Mx1 and Mx2).

232 Both DNA and RNA viruses have been known to alter the host cell metabolism to promote their
 233 replication. Such metabolic changes follow similar patterns that include an alteration of the
 234 central carbon metabolism and a redirection of the carbon supply to metabolic pathways that are
 235 crucial for replication, indeed several virus-specific signatures have been observed in previous
 236 studies(Mayer, Stöckl et al., 2019). However, due to the lack of patient cohorts and the high-level
 237 of containment required for CCHFV, knowledge about host-virus interaction has been
 238 limited(Papa, Tsergouli et al., 2017) until now. The transcriptomics data on patient materials and
 239 *in vitro* cell culture assays indicated transient dysregulation of glycolysis/gluconeogenesis after
 240 CCHFV infection, which is known to promote replication in several other RNA viruses including
 241 HIV-1, rubella virus, dengue virus, rhinovirus, hepatitis C virus (HCV), influenza virus etc
 242 (Eisenreich, Rudel et al., 2019, Fontaine, Sanchez et al., 2015, Mayer et al., 2019, Smallwood,
 243 Duan et al., 2017, Thaker, Ch'ng et al., 2019). The upregulation of glycolysis/gluconeogenesis in
 244 CCHFV infection could be mediated by the HIF-1 signaling pathway, as the HIF-targeted genes
 245 coding for the key glycolytic enzymes ENO1 and PGM2 were significantly upregulated in
 246 CCHFV infected patients during the acute phase. We therefore hypothesize that during the
 247 CCHFV infection, the stabilization and subsequent activation of HIF-1 regulates the
 248 glycolysis/gluconeogenesis that is required for virus replication. This is further supported by our
 249 previous study in which exogenous nitric oxide (NO) inhibited CCHFV *in vitro* (Simon, Falk et
 250 al., 2006) NO regulates the HIF-1 via the PI3K/Akt pathway under normoxic conditions (Sandau,
 251 Faus et al., 2000). Interestingly we have observed a similar interplay of HIF-1 signaling and

PI3K/Akt signaling in SARS-CoV-2(Appelberg et al., 2020), where NO is able to suppress SARS-CoV-2 replication *in vitro*(Akaberi, Krambrich et al., 2020). The growing interest in hypoxia-induced signaling in emerging and re-emerging viruses and its relation with NO biology is expected to lead to further insights into the complex roles of NO, and to HIF signaling as a crucial feature of NO-mediated events during acute viral diseases. Several genes were significantly downregulated in the PI3K/Akt pathway during acute CCHFV infection, indicating the dysregulation of a pathway that is critical for the cellular defense against invading pathogens.

Interestingly, an *in vitro* study in another *Bunyaviridae* virus, Rift valley fever virus (RVFV), identified the inhibition of the PI3K/Akt pathway by dephosphorylation of the AKT and Forkhead box protein O1 (FoxO1) (Popova, Turell et al., 2010). In our study we observed that the majority of the coding genes in the FoxO signaling pathway were downregulated by CCHFV, including the FOXO transcription factors FOXO1 and FOXO3, that can act as negative feedback regulators of the innate cellular antiviral response(Lei, Zhang et al., 2013). This could be due to the regulation of PI3K signaling as observed in the Epstein-Barr virus (EBV) infection (Shore, White et al., 2006). FoxO1 and FoxO3 also play an essential role in the immunometabolic dynamics and are important targets for glycolysis and gluconeogenesis (Lundell, Massart et al., 2019, Puigserver, Rhee et al., 2003).

Another critical signaling pathway that was downregulated during the CCHFV infection was AMP-activated protein kinase (AMPK) signaling that has an antiviral mechanism in addition to its role in regulating glycolysis/gluconeogenesis and FoxO1 transcriptional activity (Saline, Badertscher et al., 2019). Earlier *in vitro* studies showed that HCV inhibits the AMPK signaling pathway to promote viral replication, resulting in the downregulation of IFN-I signaling (Mankouri, Tedbury et al., 2010, Tsai, Chang et al., 2017). Interestingly, activation of the AMPK signaling pathway has been shown to inhibit the replication *in vitro* of the bunyavirus RVFV by inhibiting fatty acid synthesis (Moser, Schieffer et al., 2012). This indicates that modulation of the AMPK signaling could be an attractive therapeutic intervention for CCHFV infection through metabolic reprogramming or modulation of the IFN-I response.

The interferon signaling pathway plays a central role in innate immune responses against viruses and mediates stimulation of antiviral ISGs and secretion of pro-inflammatory cytokines. RIG-I has been recognized as the pathogen recognition receptor that recognizes CCHFV RNA, subsequently initiating a signaling cascade leading to secretion of IFN-I, mainly IFN- α and IFN- β (Spengler, Patel et al., 2015). The secreted IFN-I is further identified by the type I interferon (IFN- α/β) receptor (IFNAR) which initiates JAK-STAT signaling, leading to the stimulation of

ISGs. The protective role of IFN-I against CCHFV has been exemplified in mouse models, in which IFNAR^{-/-} or STAT-1^{-/-} mice (Bente, Alimonti et al., 2010, Zivcec, Safronetz et al., 2013) or STAT2^{-/-} hamsters (Ranadheera, Valcourt et al., 2020) showed enhanced susceptibility to CCHFV infection. Even in *in-vitro* experiments, pre-treatment of cells with IFN- α was found to be inhibitory to CCHFV (Andersson et al., 2008). Though CCHFV is inhibited by the IFN - response, not many ISGs with anti CCHFV activity have been identified apart from MxA although, ISG20 and PKR have been proposed (Andersson et al., 2004) to have anti-CCHFV activity. Nevertheless, the anti-CCHFV activity of ISGs has not been characterized. Furthermore, in the STAT-1^{-/-} mice model, CCHFV induced several IFN-stimulated genes in a delayed fashion (Zivcec et al., 2013). However, there are limited data on the effect of CCHFV on ISG stimulation in human samples. In humans, the role of the IFN response in CCHFV infection is noted by the fact that Toll-like receptor (8/9 or 3) polymorphism was shown to be associated with increased infection and fatality (Engin, Arslan et al., 2010). In the present study we mapped the ISGs induced during the acute phase of CCHFV infection compared to the recovered phase and observed that several ISGs related to the IFN-I pathway were upregulated (Figure 3a and 3b). We also observed that the ISGs with known or proposed anti-CCHFV activity, i.e. Mx1, ISG15 and ISG20 were upregulated.

Additionally, we observed significant upregulation of IFI27 (ISG12), IFITs, OASs and ISG15 (Figure 3b). IFI27 has been shown to have an antiviral effect on West-Nile virus (Lucas, Richner et al., 2015) but its impact on other viruses has not been well-studied. IFI27 was shown to distinguish between influenza and bacteria in respiratory infections (Tang, Shojaei et al., 2017) and is present at high levels in severe cases of Enterovirus 71 (EV71)-induced severe hand foot and mouth disease (Min, Ye et al., 2020), indicating its potential to be used as a biomarker. Though statistical significance was not reached, IFI27 was 1.4-fold higher in severity groups 2 and 3 compared to severity group 1. Our study observed that IFI27 was highly differentially upregulated in CCHF infection; its role in controlling the infection therefore and its possible use as a biomarker requires interrogation.

We further observed changes in the induction of ISGs during infection in a time-dependent manner *in vitro* by infecting the Huh7 cell line with CCHFV and observed several ISGs to be upregulated. Though we did not identify IFI27 in proteomics data, several ISGs including Mx1, OAS3, ISG20, ISG15, IFIT3, IFITM3, IFI16 that were upregulated in the acute phase CCHFV patient samples were observed to be also upregulated in the cell-infection model (Figure 5a-d). Among these, ISG20 has been shown to have antiviral activity against related-Bunyaviruses

(Feng et al., 2018), and we observed some degree of inhibitory activity of ISG20 on CCHFV replication (Figure 5e). Notably, Mx2 was differentially regulated in a time-dependent manner. At 24 hpi, Mx2 was significantly downregulated but it was upregulated by 48 hpi (Figure 4a, b and c), suggestive of a suppressive mechanism by the virus during early infection. The virus has evolved several mechanisms of evading the antiviral effects of the ISGs as observed in the case of ISG20 in our study, and one such mechanism is the de-ubiquitinase and de-ISGylase activity of the ovarian tumor (OTU)-protease encoded by the L1 protein (Scholte et al., 2017) that could directly inhibit ISG15 activity.

Similar to IFN-I, proinflammatory cytokines are important for effective innate immunity. CCHFV patients are known to have higher levels of plasma cytokine and chemokine compared to healthy people. Elevated levels of IL-6, IL-10 and TNF- α are most commonly associated with fatal outcomes (Ergonul, Tuncbilek et al., 2006, Korva et al., 2019, Saksida, Duh et al., 2010). A few studies have suggested that the co-elevation of IL-6 and TNF- α are strongly associated with fatality (Ergonul et al., 2006). Our study did not include samples from fatal cases and thus we performed a cytokine array and compared moderate and severe cases. Our results further confirmed the roles of TNF- α , IL-10, IL-8 and GM-CSF since these showed significant or near-significant elevation in severe cases. Korva et al., also observed significant differences in IL-10 and GM-CSF between moderate and severe cases (Korva et al., 2019). In another study by Hulva et al., changes in IL-6 and TNF- α were noted between moderate and severe cases, but no significant change in IL-8 was observed (Hülya, Yilmaz et al., 2017). Although IL-6 is often associated with disease severity and fatality, we did not observe any significant difference in IL-6 levels.

In conclusion, our study provides a comprehensive description of the host-immune response against CCHFV that can explain viral pathogenesis. The interplay of the metabolic reprogramming and the IFN-mediated host antiviral mechanism could provide attractive options for therapeutic intervention of CCHF. The specific role of the different ISGs that were regulated during the course of infections need to be studied further to provide important clues for the development of antivirals or vaccines.

Materials and Methods

Ethics statement and biosafety

348 This study was approved by the Local Research Ethics Committee of the Ankara Numune
349 Education and Research Hospital, Turkey (Protocol # 17-1338) and Regional Ethics Committee,
350 Stockholm (Dnr. 2017-/1712-31/2). All patients and/or their relatives were informed about the
351 purpose of the study and signed a consent form before collection. All the procedures were
352 performed according to the local authority biosafety guidelines and biosafety level 4 (BSL4)
353 practice.

354 **Study design, patients, and sample collection:**

355 We enrolled 18 adult patients (≥ 18 years) diagnosed with CCHF who were followed up by the
356 clinical service of Infectious Diseases and Clinical Microbiology of Sivas Cumhuriyet University
357 Hospital, Sivas, Turkey. Information about the patients was collected, including age, gender, date
358 of symptoms onset, date of hospitalization, date of discharge/death, Severity Grading System
359 (SGS) scores and severity groups, and outcome data (Supplementary Table 1). The CCHF
360 patients were divided into three groups using the SGS scores of 1, 2 and 3 (Bakir, Engin et al.,
361 2012). Blood samples were collected on the admission day (acute stage) and from the survivors
362 one year after their recovery. The samples were used for diagnosis and were sent to the CCHF
363 Reference Laboratory, Ankara, Turkey (Public Health of Turkey). For the detection of CCHFV
364 nucleic acid, a commercial real-time RT-PCR test (Altona Diagnostics[®], Hamburg, Germany)
365 was used, and for the serological diagnosis, a commercial IgM indirect immunofluorescence
366 antibody (IFA) assay (Euroimmun[®], Luebeck, Germany) was used. Patients having positive test
367 results by PCR and/or serologic analyses were diagnosed with CCHF and such patients were
368 included in the study. Peripheral blood mononuclear cells (PBMCs) were isolated by the modified
369 density gradient centrifugation method. PBMCs and plasma were sent to the Public Health
370 Agency of Sweden, Stockholm, Sweden. Serum cytokine profiling targeting 22
371 cytokines/chemokines was performed by Public Health England using methods as previously
372 described (Dowall et al) (Dowall, Graham et al., 2019). Briefly, a 22-plex customised luminex kit
373 (Merck Millipore, Darmstadt, Germany) was designed to quantitate: Eotaxin, Granulocyte-
374 Colony Stimulating Factor (G-CSF), Granulocyte Macrophage-Colony Stimulating Factor (GM-
375 CSF), Interferon-alpha 2 (IFN- α 2), Interferon-gamma (IFN- γ), Interleukin (IL)-10, IL-12, IL-15,
376 IL-17a, IL-1a, IL-9, IL-1b, IL-2, IL-4, IL-5, IL-6, IL-8, IFN- γ -Inducible Protein 10 (IP-10),
377 Monocyte Chemotactic Protein 1 (MCP-1), Macrophage Inhibitory Protein (MIP)-1a, MIP-1b and
378 Tumour Necrosis Factor alpha (TNF- α). Two quality control samples and a standard included
379 with the kit were prepared as per the manufacturers instructions. 25 μ l of each QC and standard
380 concentration and 25 μ l of serum matrix solution were added to relevant wells of a 96 well plate in

duplicate. 25µl of each patient plasma sample and 25µl of assay buffer was added to relevant wells in duplicate. Custom pre-mixed beads were sonicated and vortexed and 25µl added to each test and standard/QC well. Plates were incubated overnight at 4°C. Following incubation, plates were washed twice with 200µl wash buffer using a hand held magnet. 25µl of detection antibody was added to each well and the plates incubated at room temperature for 1hr. 25µl streptavidin-phycoerythrin was added to each well for 30 minutes at room temperature. Each well was washed twice with 200µl wash buffer using a hand-held magnet and 100µl of 10% formalin added to each well: 40% w/v formaldehyde solution (Scientific Laboratory Supplies, Nottingham England) diluted 1:9 v/v with phosphate buffered saline solution (Thermo Fisher, Loughborough, England). The plate was subjected to formaldehyde fumigation prior to removal from Biosafety level (BSL)-4 facilities. At BSL2, the plates were washed twice with 200µl wash buffer and once with 200µl sheath buffer using a hand held magnet to remove formalin, prior to addition of 150µl sheath fluid to resuspend beads. A Luminex MAGPIX instrument using Exponent software (Invitrogen, Paisley, UK) was used to obtain results in the form of MFI (median fluorescence intensity), which was converted to concentration (pg/ml) using the standard curve prepared. Plate data was accepted based on negative background wells and QC data within the expected ranges.

397 **RNA extraction and sequencing (Illumina RNAseq):**

398 Total RNA was extracted from Trizol-treated PBMC using the Direct-zol RNA Miniprep (Zymo
399 Research, CA, USA) according to the manufacturer's protocol, and the RNA quality was
400 determined by Agilent RNA 6000 Pico kit on an Agilent 2100 Bioanalyzer (Agilent
401 Technologies, Waldbronn, Germany). RNA-Seq was performed at the National Genomics
402 Infrastructure, Science for Life Laboratory, Stockholm, Sweden, as described by us
403 previously(Zhang, Ambikan et al., 2018).

404 **Data processing and transcriptomics analysis:**

405 The raw reads were first checked for quality and adapter content using the FastQC v0.11.8
406 (<https://github.com/s-andrews/FastQC>) tool. The Illumina adapter sequences found in the raw
407 reads were trimmed using the tool Cutadapt v1.18 (Martin, 2011). The mean quality score across
408 each base position in the cleaned reads was above Q30. The cleaned reads were then aligned
409 against the human reference genome version GRCh38. The reference genome was obtained from
410 the Illumina iGenomes repository. RNASeq aligner STAR v2.6.1a_08-27 was used for the
411 alignment (Dobin, Davis et al., 2013). A gene-level read count table was generated from the
412 alignment using featureCounts v1.6.3 (Liao, Smyth et al., 2014). The read count table was further

split into protein-coding genes and non-coding genes. There were 22,022 protein-coding genes. All the downstream analysis was performed on the protein-coding genes.

The raw read count data were first normalized to log2 transformed counts per million (CPM) to remove the library size effect from the R/Bioconductor package edgeR v3.28.1 (Robinson, McCarthy et al., 2010). The distribution of all samples was then visualized after reducing the dimension of the data by applying the Uniform Manifold Approximation and Projection for Dimension Reduction (UMAP) technique using R package umap v0.2.6.0 (Leland, John et al., 2018). The reduced dimensions of the data were plotted in 2D space using R package ggplot2 v3.3.2 (Wickham, 2009). Differential gene expression analysis was performed using raw read counts of remaining samples using the R/Bioconductor package DESeq2 v1.26.0 (Love, Huber et al., 2014). Genes with adjusted p-values of less than 0.05 were considered as significantly regulated. Functional analysis of the significantly regulated genes was performed using the enrichr module of the python package GSEAPY v 0.9.16 (<https://pypi.org/project/gseapy/>) (Kuleshov, Jones et al., 2016, Subramanian, Tamayo et al., 2005). The total number of protein-coding genes as background and KEGG gene-set library downloaded from Enrichr web resources were used for the enrichment test for molecular pathway analysis. Pathways having adjusted p-values of less than 0.05 were regarded as significant. Gene ontology (GO) enrichment analysis was performed using the Enrichr web resource (Kuleshov et al., 2016), and the GO biological process 2018 category was selected. GO terms with adjusted p-values of less than 0.05 were considered as significant. Redundant GO terms were removed using the online tool REVIGO (Supek et al., 2011) using the following settings: allowed similarity 0.5, GO database *Homo sapiens*, and semantic similarity measure SimRel. Heatmaps were generated using the R/Bioconductor package ComplexHeatmap v2.2.0 (Gu, Eils et al., 2016). Bubble plots, MA plots, Volcanoplots, violin plots and bar plots were created using R package ggplot2 v3.3.2. Network visualization was performed using Cytoscape v3.6.1 (Shannon, Markiel et al., 2003). Venn diagrams were constructed using the online tool InteractiVenn (Heberle, Meirelles et al., 2015).

Cells and viruses:

The CCHFV strain IbAr10200 (originally isolated from *Hyalomma excavatum* ticks from Sokoto, Nigeria, in 1966) was used in this study. The SW13- ATCC®-CCL-105™ and human hepatocyte-derived cellular carcinoma cell line Huh7 was obtained from Marburg Virology Laboratory, (Philipps-Universität Marburg, Marburg, Germany) and matched the STR reference profile of Huh7 (Rohde, Becker et al., 2019). CCHFV was propagated and titered in SW13 cells. The virus was serially diluted 10-fold and 100ul of each dilution was added to SW13 cells in a 96 well

plate. At 48 h post-infection (hpi), cells were fixed in ice-cold acetone-methanol (1:1) and stained using a rabbit polyclonal anti-CCHFV nucleocapsid antibody followed by a fluorescein isothiocyanate (FITC)-conjugated anti-rabbit antibody (Thermo Fisher Scientific, US) and DAPI (Roche, US). The number of fluorescent foci in each well was counted using a fluorescence microscope and the titer was determined. Human 293 FLP-IN TRex cells expressing CAT or ISG20 under the control of a Tet-on promoter were kindly provided by Ju-Tao Guo (Drexel University College of Medicine, Pennsylvania, USA). (Jiang, Weidner et al., 2010) The cells were propagated in DMEM supplemented with 5% Tet-negative FCS (PAA Lab, US).

***In vitro* infection assay in Huh7 cells and tandem mass tag (TMTpro) labelled reverse phase liquid chromatography mass-spectrometric (RPLC-MS/MS) analysis:**

Huh7 cells were infected with the CCHFV in triplicate, as described by us previously (Appelberg et al., 2020). Briefly, Huh7 cells were infected with CCHFV IbAr10200 at a multiplicity of infection (MOI) of 1. After 1 h of incubation (37°C, 5% CO₂) the inoculum was removed, the cells were washed with PBS, and 2 ml DMEM supplemented with 5% heat-inactivated FBS was added to each well. Samples were collected in triplicate at 24 and 48 hpi along with controls. The medium was removed, cells were washed carefully with PBS and lysed in lysis buffer, (10 mM Tris, 150 mM NaCl, 10% SDS, and protease inhibitor). After that, 4x sample buffer (ThermoFisher, US) was added and the samples were boiled at 99°C for 10 min. Protein digestion was performed in S-Trap microcolumns (Protifi, Huntington, NY) and the resulting peptides were labeled with TMTpro tags. Labeled peptides were fractionated by high pH (HpH) reversed-phase chromatography, and each fraction was analyzed on an Ultimate 3000 UHPLC (Thermo Scientific, San Jose, CA) in a 120 min linear gradient. Data were acquired on a Q Exactive HF-X mass spectrometer (Thermo Scientific, San Jose, CA) in data-dependent acquisition (DDA) mode collecting full mass spectra at 120,000 resolution in a mass range of 350 – 1400 *m/z* for a maximum injection time (IT) of 50 ms. Tandem mass spectra of the top 20 precursors were acquired with high collision energy (HCD) of 34%, resolution of 45,000 and maximum IT of 86 ms, isolating precursors with 0.7 *m/z* and applying 45s dynamic exclusion. Proteins were searched against the SwissProt human database using the search engine Mascot v2.5.1 (MatrixScience Ltd, UK) in Proteome Discoverer v2.4 (Thermo Scientific) software allowing up to two missed cleavages. The oxidation of methionine, deamidation of asparagine and glutamine, TMTpro at lysine and N-termini were set as variable modifications; while carbamidomethylation of cysteine was set as a fixed modification. The false discovery rate (FDR) was set to 1%.

Proteomics data analysis:

The raw data were first filtered to remove missing data. Proteins detected in all samples were retained for analysis resulting in 8,501 proteins in the filtered dataset. The filtered data was then normalized by applying eight different methods using R/Bioconductor package NormalyzerDE v1.4.0 (Willforss, Chawade et al., 2019). The quantile normalization was found superior to other methods and was selected for further use. Differential protein expression analysis was performed using R/Bioconductor package limma v3.42.2 (Ritchie, Phipson et al., 2015). Proteins with adjusted p-values of less than 0.05 were regarded as significant. KEGG pathway enrichment analysis of significantly regulated proteins was performed using the enrichr module of python package GSEAPY v 0.9.16.

ISG20 inhibition assay:

Tet-inducible HEK-293 FLP-IN TRex cells containing a CAT or ISG20 expression cassette were seeded on in six-well dishes and treated with doxycycline at a final concentration of 2 µg/ml and incubated for 36 h. Cells were infected with CCHFV strain IbAr10200 at an MOI of 1, and supernatants were titrated at 48 hpi by enumeration of fluorescent foci and calculation of progeny virus titers as described by us previously(Andersson et al., 2006).

RNAscope targeting IFI27 (ISG12) and CCHF:

The *RNAscope*® ISH Assays (ACD Bioscience,US) targeting IFI27 (440111, ACD Bioscience, US) and CCHFV (510621, ACD Bioscience, US) were performed as described previously.(Zhang, Svensson Akusjarvi et al., 2018) SW13 cells were infected with CCHFV (Ibar 10200 strain) at MOI of 0.1. After 1h, inoculum was replenished with fresh Leibovitz medium containing 5% FBS and incubated 48h. After infection cells were fixed 30 min using ice-cold acetone. Cells were permeabilized using 0.1% Triton x-100 for 10 min at RT prior probe incubation at 40°C of IFI27 and CCHFV probes, Amp-1, Amp-2, Amp-3 and Amp-4 Alt C for 2h, 30min, 15 min, 30 min and 15 min, respectively. Cells were further stained using DAPI and coverslip attached using ProLong Gold Antifade reagent (P10144, Thermofisher). Images were acquired using Nikon Single Point scanning confocal with 60/1.4 oil objective.

Acknowledgments: The authors would like to acknowledge support from the National Genomics Infrastructure (NGI), Science for Life Laboratory, for RNAseq and Proteomics Biomedicum; and Karolinska Institute, Solna, for LC-MS/MS analysis. The computations were performed using resources provided by SNIC through the Uppsala Multidisciplinary Center for Advanced Computational Science (UPPMAX) under Project SNIC2017-550. The microscopy part of the study was performed at the Live Cell Imaging Facility, Karolinska Institute, Sweden, supported

by grants from the Knut and Alice Wallenberg Foundation, the Swedish Research Council, the Centre for Innovative Medicine, and the Jonasson Center at the Royal Institute of Technology, Sweden. The study is funded by Swedish Research Council Grants 2018-05766 and 2017-03126 to AM and 2017-01330 to UN, PHE Grant In Aid 109509 (inc. PhD studentship[EK]) and EU-H2020 CCHFVaccine to RH.

Author contributions: Conceptualization: U.N. and A.M., Methodology: U.N. N.E. A.M., A.V., F.W., and R.H., Formal analysis: A.T.A., S.A.,E.K., S.D., B.B., S.G., J.E.R, V.M.M. and S.S., Resources: U.N., A.M., F.W., and R.H., Writing (original draft): U.N., A.T.A, and S.G., Writing (review and editing): N.E., S.A., A.V., F.W., R.H., and A.M., Visualization: U.N., A.T.A, and S.S., Supervision: U.N., A.M., A.V., N.E., F.W., R.H., Project administration: U.N., N.E. and A.M., Funding acquisition: U.N., A.M., F.W., and R.H. All authors discussed the results, commented and approved the final version of the manuscript.

Conflict of interests: The authors declare no competing interests.

The Paper Explained: The pathogenesis and host-viral interactions of the Crimean–Congo hemorrhagic fever virus (CCHFV). We have performed a global transcriptomic analysis of peripheral blood mononuclear cells from a longitudinal cohort of CCHF patients who survived and temporal untargeted proteomics analysis of CCHFV infected cells. Our results indicate that during the acute phase of CCHFV infection, the host's metabolic reprogramming towards central carbon metabolism including glycolysis/gluconeogenesis occurs. This could be regulated by the anti-viral signaling pathways that play a central role in viral replication. Therefore targeting type I interferon response through metabolic perturbation could be an attractive therapeutic intervention for CCHFV as well as aid as antiviral strategies.

Data and materials availability: All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Raw RNAseq data is available in Sequence Read Archive (SRA) with temporary id SUB8608640. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD022672. All the codes are available in GitHub (<https://neogilab.github.io/CCHF-Turkey/>)

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Figures

Fig. 1. Study design and severity group association with gene expression and cytokine profiling. (a) Overall work-flow of the data generation and analysis. (b) Sample distribution during the acute phase of infection in different severity groups as reported. (c) MA plot of differentially regulated genes during the acute phase between samples of severity group 1 and severity group 2 or 3. (d) Violin plot of significantly changed proteins as determined from ELISA assays.

Fig. 2. Differential gene expression and pathway analysis between acute and recovery phases. (a) Heatmap of Z-score transformed expression values of significantly regulated genes in the pair-wise comparisons namely Recovered vs Acute (overall), Recovered vs Acute (severity group 1), Recovered vs Acute (severity group 2). The columns represent the patient samples and their corresponding severity groups at different time points. The rows represent genes that are hierarchically clustered based on Euclidean distance. (b) KEGG pathway enrichment analysis results of genes belonging to cluster 1 of the heatmap. The bubble size and colour gradient correspond to the number of genes and adjusted P value of the enrichment test, respectively. (c) KEGG pathway enrichment results of genes belonging to cluster 2 of the heatmap. The bubble size and color gradient correspond to the number of genes and adjusted P value of the enrichment test, respectively. (d) Venn diagram of significantly up-regulated genes in Recovered vs Acute (severity group 1) and Recovered vs Acute (severity group 2) phases (e) Venn diagram of significantly down-regulated genes in Recovered vs Acute (severity group 1) and Recovered vs Acute (severity group 2) phases. (f) Gene ontology (biological process) enrichment analysis results of commonly regulated genes (882 upregulated and 569 down-regulated) from (d) and (e). the colour gradient and bubble size correspond to the gene ratio of each GO term and the adjusted P value of the enrichment test, respectively. The adjacent bar graph represents the percentage of genes upregulated or downregulated in each gene ontology term.

Fig. 3. Differentially expressed genes in interferon (IFN) signaling pathways. (a) Heatmap visualizing the expression pattern of IFN-signaling genes (including ISGs) that are significantly different between the recovered and acute phases. The columns represent the patient samples and

their corresponding severity groups at different time points. The rows represent genes hierarchically clustered based on Euclidean distance. The row annotation indicates the differential expression of genes (DEG) and presence or absence of genes in the selected GO terms. The names of genes involved in the cellular response to IFN- γ (GO:0071346) and the IFN-I signaling (GO:0060337) are printed. **(b)** MA plot of differentially regulated genes between the recovered and acute phases. ISGs are marked. **(c)** RNAscope analysis targeting IFI27 genes in infected and non-infected cells. **(d)** Network type visualization of genes belonging to GO:0071346 and GO:0060337. The edges indicate the presence of the corresponding gene in the gene-ontology term. The node size and colour gradient correspond to the adjusted P value of differential expression analysis and the log2 fold change, respectively.

Fig. 4. LC-MS/MS based proteomics analysis in CCHFV infected Huh7 cells. **(a)** General work-flow showing the sample preparation and data generation for the proteomics experiment. **(b)** Significant pathways identified by mapping proteins regulated over time to the KEGG database. The pathways are grouped into two: the first 29 pathways are metabolism related and the last 16 pathways are related to organismal systems and environmental information processing. The bubble size and colour gradient correspond to the number of proteins and adjusted P value of the enrichment test, respectively. The bar graphs represent the percentage of proteins upregulated or downregulated in each pathway as per the corresponding comparisons.

Fig. 5. Temporal dynamics of interferon stimulating genes (ISGs). **(a)** Heatmap of Z-score transformed expression values of proteins belonging to the cellular response to IHN- γ (GO:0071346) and the IFN-I interferon signaling pathway (GO:0060337) gene ontology terms. The columns represent the different samples and their corresponding treatment conditions and time points. The rows represent proteins hierarchically clustered based on Euclidean distance. The row annotation indicates the presence or absence of proteins in the corresponding GO terms and log 2-fold change values in the proteomics and transcriptomics (recovered -vs- acute) experiments. **(b)** Volcano plot of ISGs visualizing the expression status of 24 h Mock and Infected (24 hpi) samples. The size and colour gradients of the dots correspond to the adjusted P values of differential expression analysis and the log2 fold change, respectively. **(c)** Volcano plot of ISGs visualizing the expression status of 48 h Mock and Infected (48 hpi). The size and colour gradient of the dots correspond to the adjusted P values of differential expression analysis and log2 fold change, respectively. **(d)** Volcano plot of ISGs visualizing the expression status of 24 hpi and 48 hpi. The size and colour gradient of the dots correspond to the adjusted P value of differential expression analysis and the log2 fold change, respectively. **(e)** HEK293-FLP-IN T-

Rex cells that conditionally express ISG20 or human CAT protein were treated with doxycycline or left untreated for 36 h, followed by infection with CCHFV at an MOI 1 for 48hours. Cell-free culture supernatants were harvested and progeny viral titers were determined by fluorescent focus-forming assay. Columns show means $\pm$ the standard deviations ($n = 2$). A two-tailed paired Student t test was performed to compare titers of untreated and doxycycline-treated cells.

Fig. 6. Potential host-cellular, immune response and metabolic reprogramming in CCHFV infection. The figure is created with BioRender.com.

Supplementary Materials

Supplementary Table S1. The CCHF patient characteristics.

Supplementary Figure S1: The heterogenous gene-expression profile was further refined by hierarchical clustering analysis of the top 500 high variable genes

Supplementary Figure S2. The levels of all of the rest 18 soluble marker.

Supplementary Figure S3: UMAP clustering of the acute phase and recovered phase samples.

Supplementary Figure S4: Overlap of the pathways identified between patients based transcriptomics data and in vitro cellular data.

Supplementary figure S5. Potential pathways regulated by CCHFV during acute phase of infection

Supplementary Data File 1: The DGE profile for the acute phase compared to the recovered phase in all patients showed an upregulation of 2891 genes and a downregulation of 2738

Supplementary Data File 2: The pathways that are significantly modulated by the upregulated and downregulated genes.

a. Severity group 1, 2, and 3

b.

c.

d.

a.

b.

c.

d.