SUPPLEMENTARY MATERIAL

Severe SARS-CoV-2 placenta infection can impact neonatal outcome in the absence of vertical transmission.

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The authors have declared that no conflict of interest exists.

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SUPPLEMENTARY METHODS

Patient data. Pregnant women with COVID-19 delivering between March 12th and April 23rd 2020 at Fondazione IRCCS Ca' Granda – Ospedale Maggiore Policlinico (Milan, Italy) were retrospectively identified via the electronic health record. Controls were women undergoing third trimester delivery in the same period with clinically indicated placental pathology examination (at physician´s judgment) but tested negative for SARS-CoV-2 infection by nasopharyngeal swab PCR. Formalin-fixed paraffin-embedded (FFPE) placental tissue samples were collected from all women included in the study. Histopathological evaluation was performed by two expert pathologists (FMC and GAC) according to the Amsterdam Placental Workshop Group Consensus Guidelines (1). Lung samples from SARS-CoV-2 positive patients were collected post mortem at the Department of Pathology, University of Basel (Switzerland).

RT-PCR. Quantitative RT-PCR for SARS-CoV-2 on placenta and lung tissue samples were performed using two different assays. Allplex™ 2019-nCoV Assay (Seegene Inc, South Korea), which targets E, RdRp and N genes, and TaqMan™ 2019-nCoV Assay Kit-v2 (Thermofisher Scientific, USA), which targets N, ORF1ab and S genes. According to the manufacturer's instructions, samples with two or more positive targets were considered as SARS-CoV-2 positive. All samples were equally classified by the 2 assays. Only results from the TaqMan™ 2019-nCoV Assay Kit-v2 are shown in Table S3 and S4.

In situ analyses. Viral RNA was visualized in FFPE tissue sections using the RNAscope® (Advanced Cell Diagnostics USA; Newark, CA) *in situ* hybridization assay as previously described (2). Results were expressed in density values (number of signals per mm^2 of tissue). Apoptosis was analyzed by immunohistochemistry (IHC) using the anti-cleaved caspase-3 (Asp175) rabbit polyclonal antibody from Cell Signaling Technologies (cat#9661) and quantified by image analysis.

Gene expression. RNA expression profiling through Nanostring was performed by Pangea Oncology, with an in-house built panel of 90 genes containing the genes specified in the three gene signatures of interest: cytotoxic cells, macrophages and the IFNɣ signature defined by Ayers and colleugues (Table S5) (3). We normalized Nanostring counts with NSolver© software. We adjusted for background noise thresholding by the maximum of counts of the negative controls and the geometric mean of the counts of the positive controls. Next, expression counts were log2

transformed. Enrichment score of the three aforementioned gene signatures was calculated according to the following formula:

$$\frac{patientZ_i - min(patientsZ)}{max(patientsZ) - min(patientsZ)}$$

where μ refers to average σ to standard deviation and *patientsZ* is defined as follows:

$$\frac{\sum_{n-i}^{i} \blacksquare \frac{gene_i - \mu(gene\ across\ patients)}{\sigma(gene\ across\ patients)}}{n\ (signature\ genes)}$$

Samples were aggregated according to the enrichment score of the three signatures using the ward method, through Scipy cluster package (4). The number of clusters for downstream analyses was set to three.

Statistics. Statistical analyses were carried out using SPSS statistics 24 software. Comparison of continuous variables was carried out with the non-parametric Mann-Whitney U test. For analysis of categorical variables, we used the chi-square test or Fisher's exact test. For gene expression, all statistical analysis, including Mann-Whitney test and P-value adjustment (False Discovery Rate Benjamini & Hochberg method) were performed through Python SciPy library (4). Statistical significance was accepted at the conventional two-sided $P < 0.05$ thresholds.

SUPPLEMENTAL REFERENCES.

1. Khong TY, et al. Sampling and definitions of placental lesions Amsterdam placental workshop group consensus statement. Arch Pathol Lab Med. 2016;140(7):698-713.

2. Serna G, et al. Fusobacterium nucleatum persistence and risk of recurrence after preoperative treatment in locally advanced rectal cancer. Ann Oncol. 2020;31(10):1366-1375.

3. Ayers M, et al. IFN-γ-related mRNA profile predicts clinical response to PD-1 blockade. J Clin Invest. 2017;127(8):2930-2940.

4. Virtanen P, et al. SciPy 1.0: fundamental algorithms for scientific computing in Python. Nat Methods. 2020;17(3):261-272.

Table S1. Summary of placental histopathology in COVID+ and COVID- cohorts.

Feature	Patient 1	COVID+ (n=21)	COVID- (n=16)	Odds ratio vs COVID-	*P*-value vs COVID-
Fetal vascular malperfusion (FVM), any	0	4 (19)	2 (12)	1.89	0.68
Delayed villous maturation (DVM)	0	1 (5)	4 (25)	0.19	0.14
Chorangiosis	0	2 (9)	4 (25)	0.37	0.37
Maternal Vascular malperfusion (MVM), any	1	6 (29)	2 (12)	3.20	0.42
Central villous infarction	1	1 (5)	0 (0)	ND	1
Peripheral villous infarction	1	1 (5)	0 (0)	ND	1
Acute inflammatory pathology					
Fetal inflammatory response stage 2	0	0 (0)	3 (19)	0	0.07
Maternal inflammatory response stage 2	0	2 (9)	4 (25)	0.27	0.37
Chronic inflammatory pathology					
Villitis, high grade	1	1 (5)	0 (0)	ND	1
Villitis, low grade	0	3 (14)	3 (19)	0.83	1
Other placental findings					
increased perivillous fibrin	1	4 (19)	1 (6)	4,00	0.36
MPFD with trophoblast damage	1	1 (5)	0 (0)	ND	1
perivillous calcification	1	8 (38)	6 (37)	0.97	1
intravillus calcification	1	3 (14)	4 (25)	0.56	0.67

MPFD, massive perivillous fibrin deposition. ND, not determined.

Table S2. Characteristics of maternal and neonatal outcomes in women with positive nasopharyngeal swab (n=21) according to SARS-CoV-2 placenta status.

		SARS-CoV-2 PCR		
		NEG	POS	P
BMI		25 (24-33)	27.5 (23-30)	.84
Gestational age at diagnosis		36 (32-39)	37 (37-38)	.92
Gestational age at delivery		38 (36-40)	38 (37-39)	.47
Ethnicity	Caucasian	6	5	.20
	Asian	0	3	
	Hispanic	5	2	
Indication for nasopharyngeal swab	Screening	5	4	.57
	Signs and symptoms	6	6	
Sign and symtoms at admission	No	5	4	.57
	Yes	6	6	
Complications during hospitalization	No	8	7	.63
	Yes	3	3	
Pregnancy complications	No	7	5	.42
	Yes	4	5	
Fetal complications	No	11	8	.21
	Yes	0	2	
Premature delivery	No	7	8	.37
	Yes	4	2	
Mode of delivery	Vaginal	8	3	.09
	Cesarean	3	6	
Labour	Spontaneous	5	0	0.1
	Induction	4	4	
Type of caesarean section	Elective	1	3	1
	Emergency	2	3	
APGAR 1	1	0	1	.24
	8	2	0	
	9	9	8	
APGAR 5	4	0	1	.41
	8	1	0	
	9	1	0	
	10	9	8	
Placental weight		480 (390-504)	502,5 (440-580)	.39
Neonatal weight		3140 (2500-3635)	2990 (2770-3200)	.46
pH		7.3 (7.30-7.35)	7.28 (7.25-7.37)	.77

Dichotomous variables were expressed as count and continuous variables as median (interquartile range).

Table S3. Characteristics of post-mortem lung patients.

GEcode	Basic demographics				Hospitalization until death (days)	Intubation	PCR Testing source		SARS-CoV2
	Age	Gender	Weight (Kg)	Height (cms)			Nasopharyngeal swab	post mortem tissue	Ct* (N/ORF1/S)
E1469	85	M	71	164	5	no	positive	positive	15.25/17.97/15.38
E1470	95	M	64	166	3	no	positive	positive	16.43/18.89/16.07
E1471	74	M	91	185	3	no	positive	positive	18.17/19.43/16.22
E1473	71	M	79	180	0	no	positive	positive	17.23/18.93.17.25

*** qRT-PCR** of **N/ORF1/S genes cycle threshold (Ct) values from the TaqMan™ 2019-nCoV Assay Kit v2 (Thermofisher Scientific, USA).** Lower cycle thresholds (Ct) indicate higher viral loads.

Table S4. Tissue samples used in the study and analyses performed.

Patient ID	GEcode	Tissue	Clinical Class	Tissue Class	Ct (N/ORF1/S)	Signal/mm^2
1	E1501	PLACENTA			13/14.5/14	4,2
1	E1503	PLACENTA				4,2
3		PLACENTA			29/30/31	-0,3
24	E1423	PLACENTA			29/30/31	0,3
4		PLACENTA			30/32/31	0,0
19		PLACENTA			34/32/32	-0,2
27		PLACENTA			34/32/32	0,0
17		PLACENTA			30/31/33	0,1
8		PLACENTA			34/34/34	0,0
9	E1479	PLACENTA			34/32/35	-0,2
18	E1493	PLACENTA			34/35/36	0,0
2	E1415	PLACENTA			40/40/40	-0,3
6	E1485	PLACENTA			34/40/40	-0,2
7		PLACENTA			40/40/40	-0,3
10	E1416	PLACENTA			40/40/40	-0,3
11	E1481	PLACENTA			40/40/40	-0,4
13		PLACENTA			40/40/40	0,1
14		PLACENTA			40/40/40	0,0
16		PLACENTA			40/40/40	-0,1
21		PLACENTA			40/40/40	-0,1
20		PLACENTA				0,0
22		PLACENTA				0,0
5	E1414	PLACENTA			40/40/40	-0,1
23		PLACENTA			40/40/40	0,7
15		PLACENTA				-0,7
25	E1424	PLACENTA				-0,3
28	E1425	PLACENTA				-0,2
31	E1427	PLACENTA				0,2
32	E1496	PLACENTA				-0,2
35	E1428	PLACENTA				0,1
37	E1500	PLACENTA				0,0
38	E1429	PLACENTA				0,0
26		PLACENTA				
30		PLACENTA				
33		PLACENTA				
34		PLACENTA				
36		PLACENTA				
39		PLACENTA				
40	E1469	LUNG			15/18/15	3,7
41	E1470	LUNG			16/19/16	
42	E1471	LUNG			18/19/16	2,8
43	E1473	LUNG			17/19/17	

Patient ID: study patient identification number. GEcode: Gene expression code (only for samples undergoing gene expression analysis). Clinical class: dark red, mother with positive nasopharyngeal swab by qRT-PCR; green, mother with negative nasopharyngeal swab by qRT-PCR. Tissue class: black, placentas highly positive by qRT-PCR (Ct below 30 in at least two out of the three target genes analysed) and/or RNA-ISH (density >2 log10); dark grey, placentas weakly positive by qRT-PCR (Ct between 30 and 39) and/or RNA-ISH (density between 1 and 2 log10); light grey, placentas negative by qRT-PCR (Ct ≥40) and/or RNA-ISH (density >1 log10). Ct: qRT-PCR **of N/ORF1/S** genes cycle threshold (Ct) values from

the TaqMan™ 2019-nCoV Assay Kit v2 (Thermofisher Scientific, USA). Signal/mm^2: density of SARS-CoV-2 signals per square mm of tissue by RNA-ISH. Caspase: percentage of activated caspase-3-stained tissue.

Table S5. List of genes included in the signatures.

Gene	Signature
CXCL10	IFNg signature
CXCL11	IFNg signature
CXCL9	IFNg signature
IFNg	IFNg signature
GZMB	Cytotoxic cells (NK+CD8 Tcell)
CD8A	Cytotoxic cells (NK+CD8 Tcell)
GZMA	Cytotoxic cells (NK+CD8 Tcell)
PRF1	Cytotoxic cells (NK+CD8 Tcell)
CTSK	Macrophages
MSR1	Macrophages
CCL7	Macrophages
CD163	Macrophages
CD68	Macrophages
CD163	Macrophages
CSF1	Macrophages

Supplementary Figures

Figure S1. Morphological features of severe injured placenta from Patient 1. The intervillous space of the placental parenchyma is occupied by massive fibrin deposition associated with erythrocytes, cellular debris, neutrophilic granulocytes, lymphocytes and calcification. The syncytiotrophoblast layer of the villi is variably smudged appearing and necrotic; the villi are close together with collapse of the intervillous maternal blood space and necrotic changes, loss of trophoblast nuclear staining, and diffusely ghost villi

Figure S2. SARS-CoV-2 placenta infection and apoptosis. (A) In this figure we show representative images of the virus load by SARS-CoV-2 in situ hybridization (red signal) and apoptosis levels by activated caspase-3 immunohistochemistry (brown signal) in the severe injured placenta from Patient 1 (E1501), a clinically positive / PCR negative placenta (E1485), and a clinically positive / PCR positive placenta (E1479). Scale bar: 100um. (B) Correlation between SARS-CoV-2 load and apoptosis by immunohistochemistry in placental tissues. X-axis, SARS-CoV-2 RNA *in situ* hybridization density (log10). Y-axis, % of activated caspase-3-stained area. High levels of apoptosis are induced in two independent samples from patient 1 with high viral load (black dots) but not in samples with undetectable or weakly detectable virus.

Figure S3. SARS-CoV-2 infection in placental and lung tissues. In this figure we show images of the severe injured placenta (E1501) and a post-mortem lung sample (E1469) stained with SARS-CoV-2 and SARS-CoV-2 Sense probes detecting the S gene encoding the spike protein and the antisense strand of the S gene, respectively. Red staining indicates the presence of the virus. A probe recognizing the dapB gene was used as negative control (NEG CTRL). Results indicate that despite the high viral load, the virus is not replicating in the placental and lung tissues. Scale bars: Placenta, 5um; Lung, 250um.

Figure S4. (A) Box plots showing the distribution of enrichment scores of Cytotoxic cells (upper left), Macrophages (upper right), IFNɣ signature (upper middle), CD8A (bottom left), CD68 (bottom right), and CD163 (bottom middle) across sample groups of interest, based on SARS-CoV2 status, depicted below. Results of Mann-Whitney U test are shown as an asterisk if P-value < 0.05 or n.s. otherwise. (B, C) Volcano plot comparing placenta and lung samples (B) and intermediate and low inflammation samples (C). Each dot represents a gene and has been colored according to Mann Whitney U test results and the log2 Fold Change (FC). Significantly over-expressed genes (adjusted P-value < 0.05 and FC > 0) are colored red and significantly under-expressed genes (adjusted P-value < 0.05 and FC < 0) are colored blue; non-significant differentially expressed genes are colored grey. A dashed line represents the significance threshold, adjusted P-value 0.05. The top-5 significantly over- and under- expressed genes have been highlighted.

Figure S1

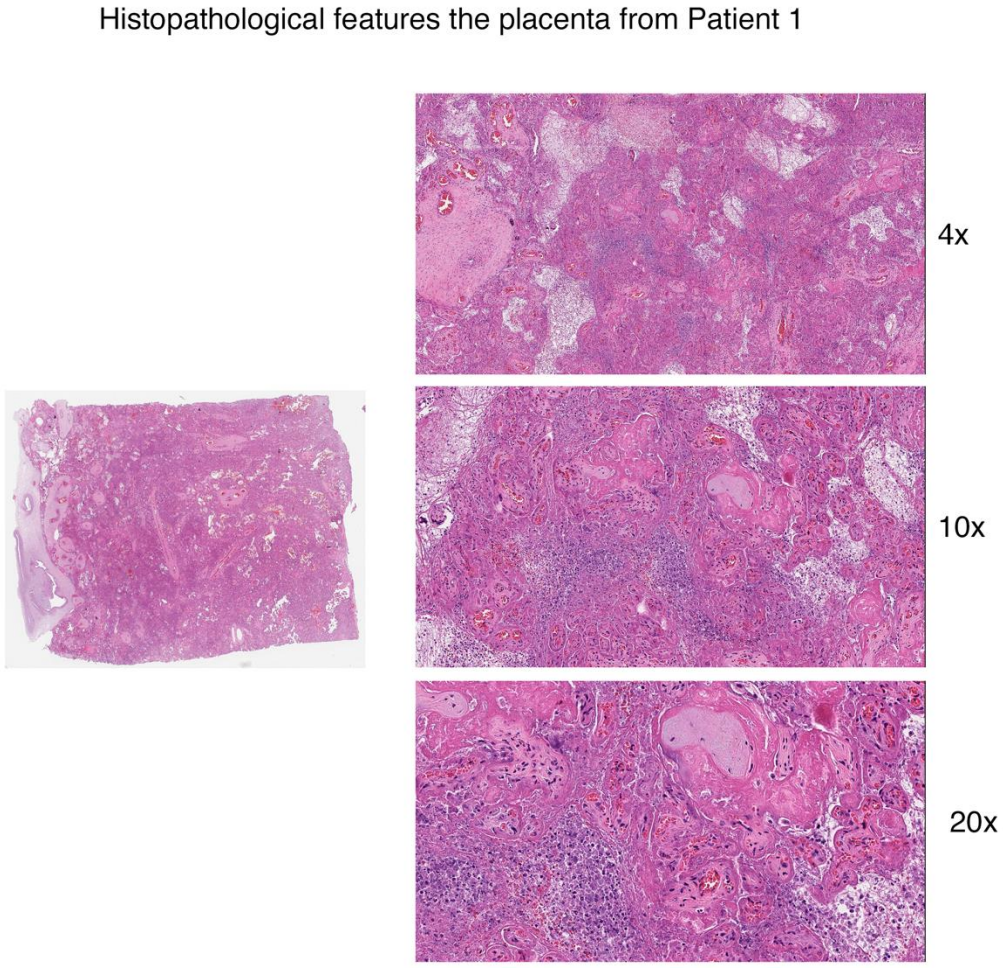

Histopathological features the placenta from Patient 1
4x
10x
20x

Figure S2

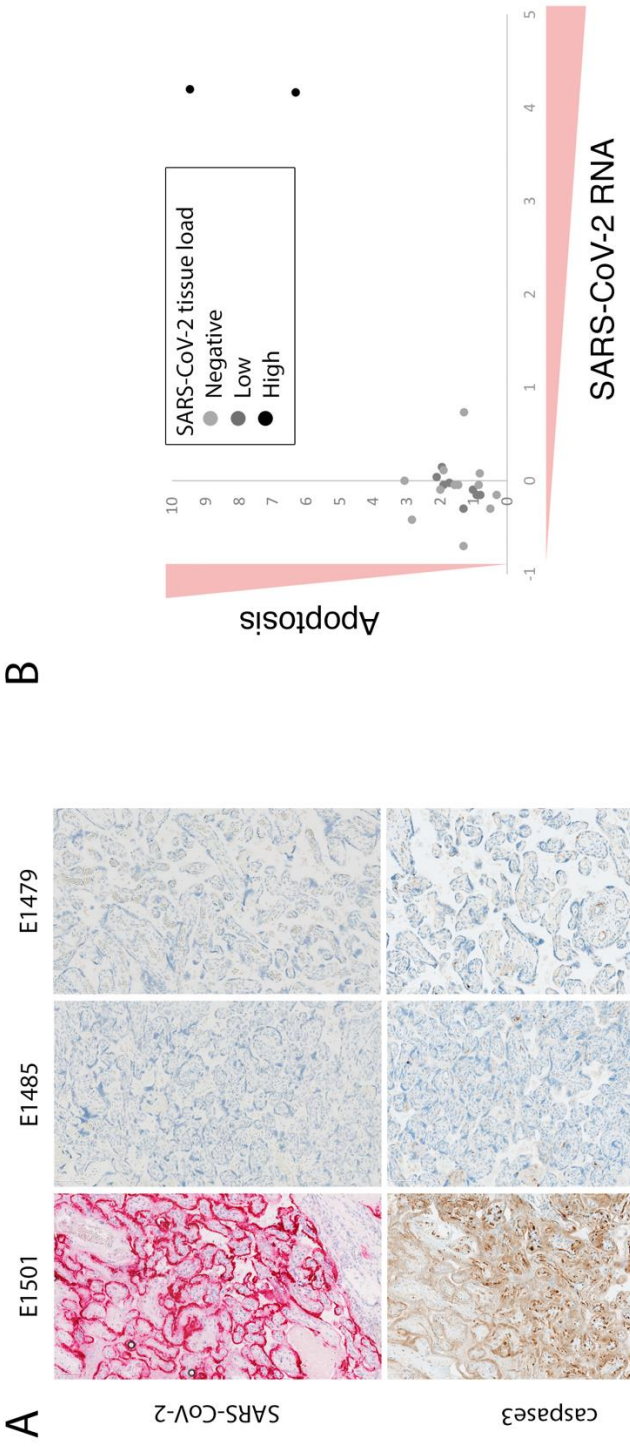

A
SARS-CoV-2
caspase3
E1501
E1485
E1479
B
Apoptosis
SARS-CoV-2 RNA
SARS-CoV-2 tissue load
Negative
Low
High

Figure S3

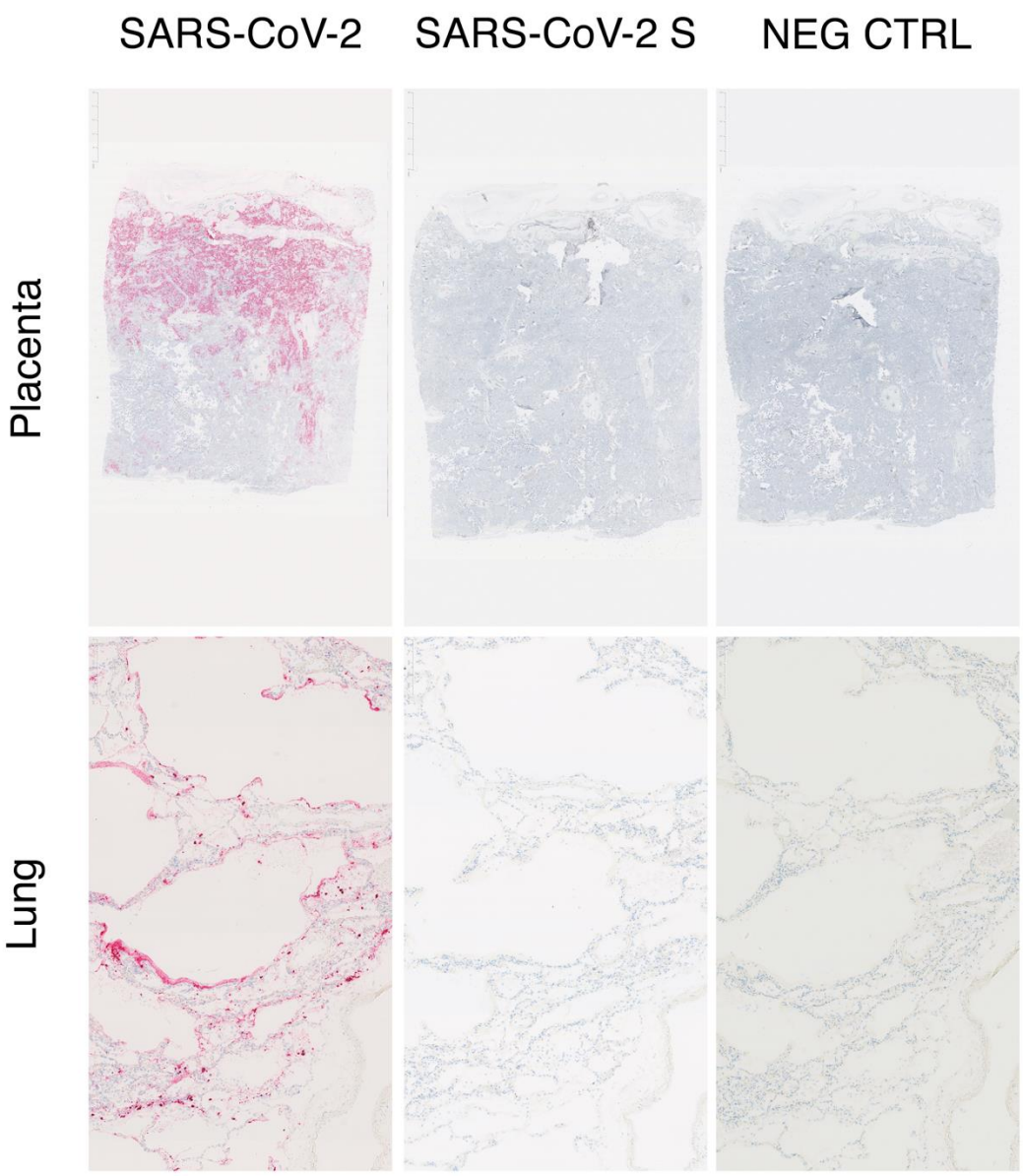
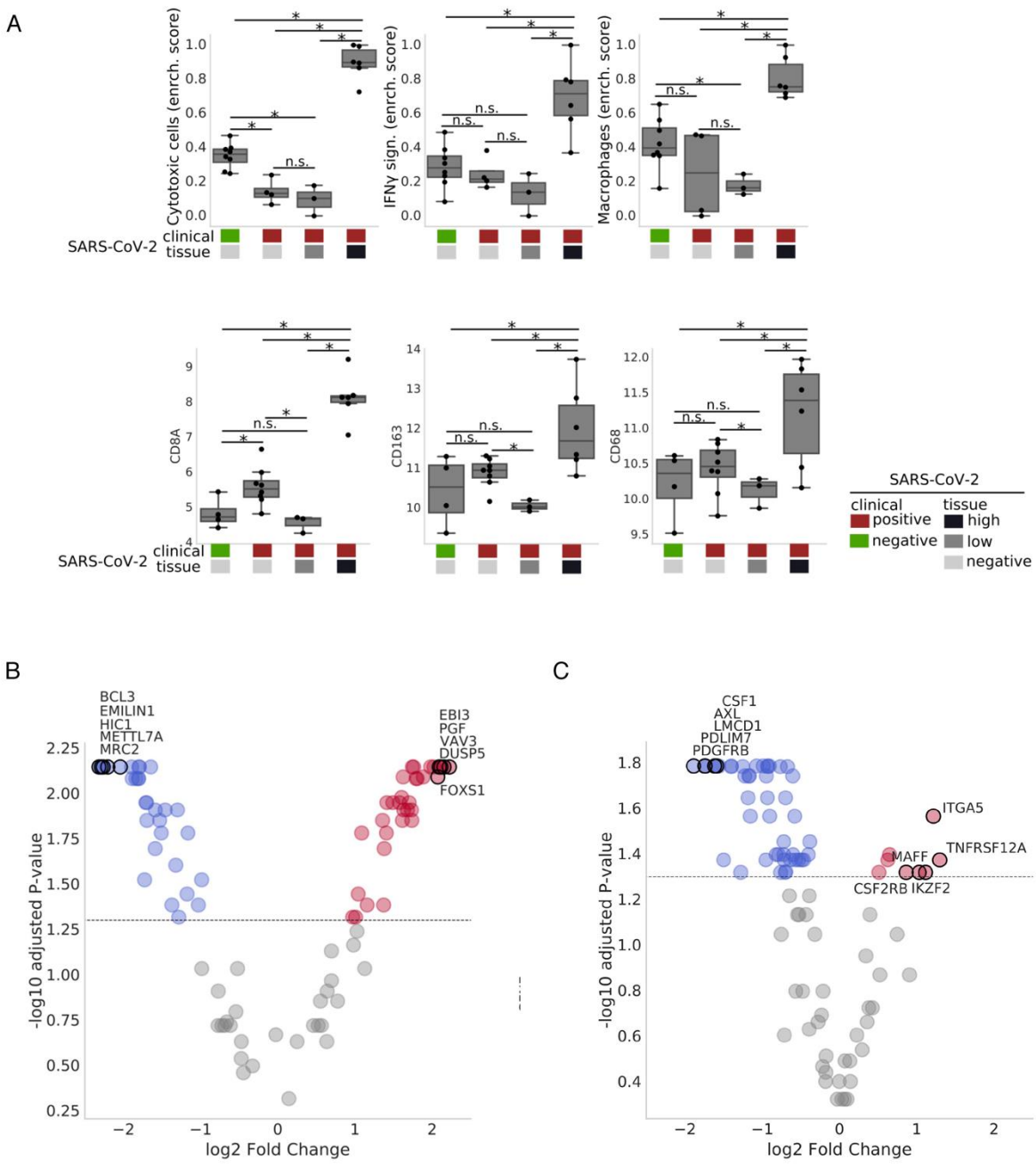


Figure S4


A
Cytotoxic cells (enrch. score)
IFNγ sign. (enrch. score)
Macrophages (enrch. score)
CD8A
CD163
CD68
SARS-CoV-2
clinical
tissue
n.s.
SARS-CoV-2
clinical
positive
negative
tissue
high
low
negative
B
BCL3
EMILIN1
HIC1
METTL7A
MRC2
EBI3
PGF
VAV3
DUSP5
FOXS1
-log10 adjusted P-value
log2 Fold Change
C
CSF1
AXL
LMCD1
PDLIM7
PDGFRB
ITGA5
TNFRSF12A
MAFF
CSF2RB
IKZF2
-log10 adjusted P-value
log2 Fold Change