

# Analysis of sc-RNA-seq of colorectal cancer

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colorectal cancer, Seurat, clustering, doublets

## Introduction

The immunotherapy for colorectal cancer is effective only for certain types of tumors. thus there has been extensive multiomics studies on molecular landscape of colorectal cancer (CRC) that allowed the classification of cells from colorectal cancer to consensus molecular subtypes (CMS). Characterisation of tumor complexity using single-cell transcriptome analysis can help to dissect molecular landscape by cellular component, which can offer a deeper understanding of tumor heterogeneity. Presence of certain types of cells like for example cancer-associated fibroblasts, which are the hallmarks of the mesenchymal phenotype CMS4 are associated with poor survival rate. (1)

Many current anticancer therapies target nontumor components such as extracellular matrix, immune system and vascular system. Thus, identifying cellular components of the tumor landscape is vital to choosing viable therapy.

In this article we analyze single-cell RNA sequencing (sc-RNA-seq) data to try to understand and classify cellular components of colorectal cancer. We we conduct our analysis according to current best practices of working with sc-RNA-seq data. (2).

## Methods

**The dataset.** The raw scRNA-seq data analyzed in this article comes from a study conducted by the Molecular Digestive Oncology Unit Department of Oncology in Katholieke Universiteit Leuven in 2018. The dataset named KUL3 was later used in a bigger study (Lee et al). Samples come from 6 Belgian patients diagnosed with CRC who underwent surgery without previous treatment. After surgical resection samples from tumor core, tumor border, and adjacent non-malignant colon tissue from the same resection specimen were collected and immediately transferred for tissue preparation. Half of the tissues were subjected to single cell isolation and RNA-seq of coding RNA. Fresh single-cell suspensions were loaded into the Chromium system (10x Genomics) targeting 5,000 cells. Sequencing data were aligned to the human reference genome (GRCh38) and processed using CellRanger 2.1.0 pipeline (10x Genomics). Further sequencing protocol information as well as raw data are available in the ArrayExpress under the accession code E-MTAB-8410 <sup>1</sup>.

The dataset downloaded from Single Cell Expression Atlas contained 52609 cells. In the analysis we used only samples assigned to 6 adult individuals with subsequent id KUL01,

	Sample	Sample size	Individual	Sex	Age (years)	Organism part	Sampling site
1	Sample1c	2992	KUL01	female	81	caecum	normal tissue
2	Sample1b	3328	KUL01	female	81	caecum	tumour border
3	Sample1a	2495	KUL01	female	81	caecum	tumour core
4	Sample2c	4015	KUL19	female	86	rectosigmoid junction	normal tissue
5	Sample2b	3920	KUL19	female	86	rectosigmoid junction	tumour border
6	Sample2a	2253	KUL19	female	86	rectosigmoid junction	tumour core
7	Sample3c	2644	KUL21	female	50	sigmoid colon	normal tissue
8	Sample3b	2209	KUL21	female	50	sigmoid colon	tumour border
9	Sample3a	1705	KUL21	female	50	sigmoid colon	tumour core
10	Sample6c	590	KUL28	male	52	sigmoid colon	normal tissue
11	Sample6b	225	KUL28	male	52	sigmoid colon	tumour border
12	Sample6a	1149	KUL28	male	52	sigmoid colon	tumour core
13	Sample8c	315	KUL30	male	84	ascending colon	normal tissue
14	Sample8b	386	KUL30	male	84	ascending colon	tumour border
15	Sample8a	2537	KUL30	male	84	ascending colon	tumour core
16	Sample9c	360	KUL31	male	85	sigmoid colon	normal tissue
17	Sample9b	1506	KUL31	male	85	sigmoid colon	tumour border
18	Sample9a	1855	KUL31	male	85	sigmoid colon	tumour core

Fig. 1. Experiment design and information about samples

KUL19, KUL21, KUL28, KUL30, KUL31, which consisted of 34484 cells. Further information about samples can be found in Figure 1.

**Filtering the raw gene expression matrix.** We analyzed the raw gene expression matrix from the CellRanger pipeline using the Seurat R package which turned out to be superior to the RCAv2 R package which couldn't manage to cluster cells using inbuilt functions.

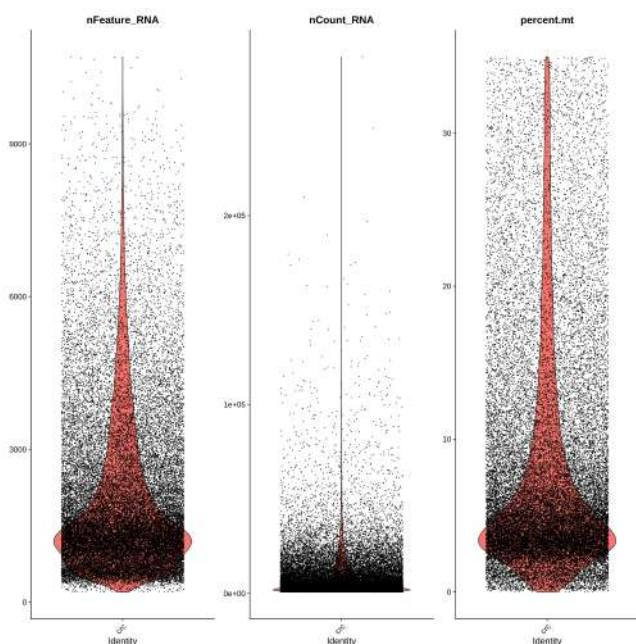
The raw gene expression matrix was loaded and used to create the Seurat object. Then the expression matrix has been filtered following the criteria stated in Lee et al. The cells with more than 1000 unique molecular identifiers (nCount\_RNA > 1000), more than 200 and less than 6000 genes (nFeature\_RNA > 200 and nFeature\_RNA < 6000) and less than 20% of mitochondrial gene expression in UMI counts.

Then we performed data normalization using the LogNormalize method implemented in NormalizeData function in Seurat, which normalizes the feature expression measurements for each cell by the total expression, then multiplies it by the scale factor (we used the default 10 000) and finally log-transforms the result. (1)

**Feature selection.** The next step was finding the subset of features that exhibit high cell-to-cell variation in the data in order to only focus on those highly variable genes in further analysis. Research shows that this type of feature selection can help to amplify biological signals in single-cell datasets. The feature selection was done using an algorithm implemented in FindVariableFeatures function in Seurat package with following parameters: selection method as "vst", return 2000 features, feature mean values between 0.0125 and 3 and feature dispersion greater than 3.

Top 10 most variable features

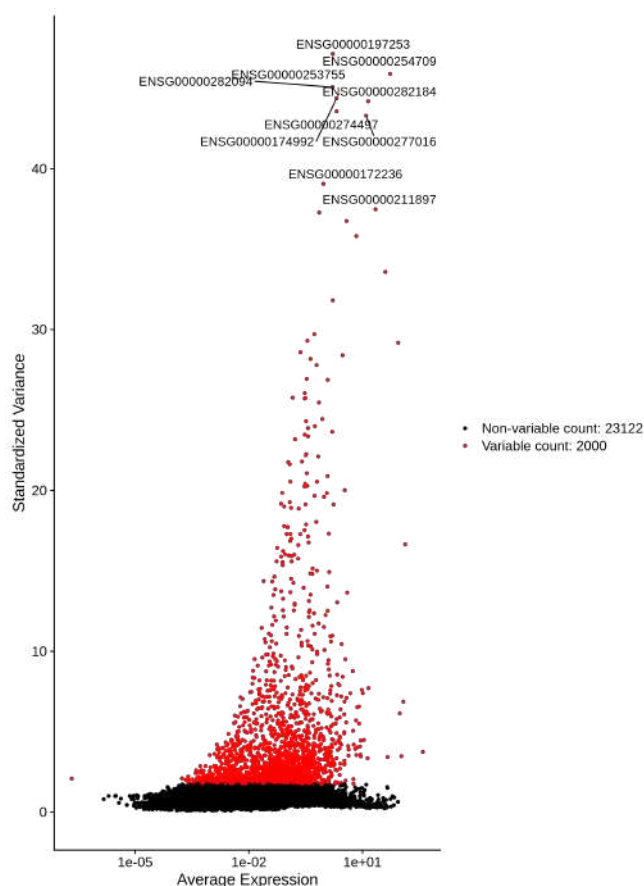
<sup>1</sup><https://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-8410/>



**Fig. 2.** the QC metrics

1. ENSG00000197253 (*TPSB2*) - Tryptase Beta 2
2. ENSG00000254709 (*IGLL5*) - Immunoglobulin Lambda Like Polypeptide 5
3. ENSG00000253755 (*IGHGP*) - Immunoglobulin Heavy Constant Gamma P (Non-Functional)
4. ENSG00000282094 (*IGHGP*) - Immunoglobulin Heavy Constant Gamma P (Non-Functional)
5. ENSG00000174992 (*ZG16*) - Zymogen Granule Protein 16
6. ENSG00000282184 (*IGHG3*) - Immunoglobulin Heavy Constant Gamma 3 (G3m Marker)
7. ENSG00000274497 (*IGHG2*) - Immunoglobulin Heavy Constant Gamma 2 (G2m Marker)
8. ENSG00000277016 (*IGHG4*) - Immunoglobulin Heavy Constant Gamma 4 (G4m Marker)
9. ENSG00000172236 (*TPSAB1*) - Tryptase Alpha/Beta 1
10. ENSG00000211897 (*IGHG3*) - Immunoglobulin Heavy Constant Gamma 3 (G3m Marker)

We can observe that genes coding constant regions of immunoglobulin heavy chains (*IGHG2*, *IGHG3*, *IGHG4*) as well as other immunoglobulin (*IGHGP*, *IGLL5*) have varying levels of expression in the cells. This is expected as we are dealing with cancer and normal cells which are characterized by different levels of immunoglobulin expression (usually higher in cancer cells) (3). The other gene with differing expression levels between cells is tryptase (*TPSB2*, *TPSAB1*) which is a major neutral protease in mast cells and is secreted



**Fig. 3.** Most variable features

upon activation-degranulation response of these cells. Mast cells accumulate in the tumors and their microenvironment during disease progression (4) so varying levels of tryptase gene expression between cancer and normal cells is expected. The ZG16 gene is associated with mucus-secreting cells, especially Intestinal goblet cells and is one of the most significantly down-regulated genes in colorectal cancer tissues (5).

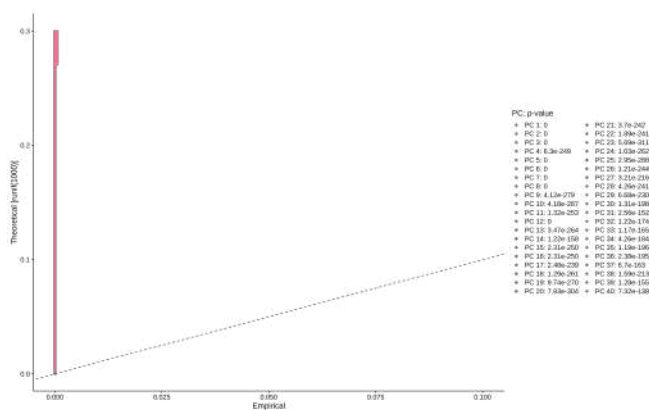


Fig. 4. Significance of top 40 PCs

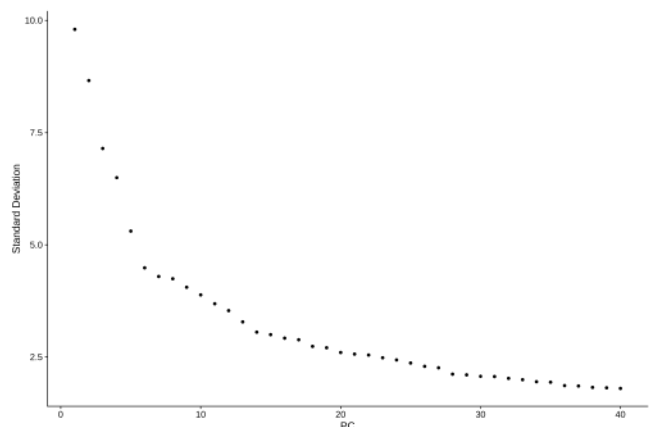


Fig. 5. Elbow plot of top 40 PCs

**Dimentional reduction.** Next step in pre-processing our data is scaling prior to dimensional reduction techniques. We use the algorithm implemented in the ScaleData function from the Seurat package. This step assigns equal weight to the genes so that in downstream analysis the highly expressed ones do not completely dominate the signal. After that the data is ready for dimension reduction techniques. We perform PCA using selected highly variable features.

To determine the "dimensionality" of the dataset we test which PCs are statistically significant using Jackstrow and ScoreJackstrow functions with the first 40 PCs.

However as seen on Figure 4 p-values of all 40 PCs are significant and very close to 0 which suggests that high number of PCs significantly explain the variance of the dataset, thus reducing dimensionality by choosing the few top PCs might result in loss of significant biological signaling reflected in the data.

After analyzing the Elbow plot Figure 5 we decided to choose the first 28 PCs for further analysis. The choice was substantiated by further clustering simulations using a greater number of dimensions which resulted in a high number of clusters. This high of a number was unnecessary as assigning cell types to clusters is a difficult problem that requires prior knowledge about cell markers and gene expression in different cell types. Thus we decided to choose a lower number of dimensions to focus on more global expression patterns in the dataset rather than trying to capture more delicate differ-

Multiplet Rate (%)	# of Cells Loaded	# of Cells Recovered
~0.4%	~825	~500
~0.8%	~1,650	~1,000
~1.6%	~3,300	~2,000
~2.4%	~4,950	~3,000
~3.2%	~6,600	~4,000
~4.0%	~8,250	~5,000
~4.8%	~9,900	~6,000
~5.6%	~11,550	~7,000
~6.4%	~13,200	~8,000
~7.2%	~14,850	~9,000
~8.0%	~16,500	~10,000

Fig. 6. Multiplet rate is linearly dependent on the amount of loaded cells

ences.

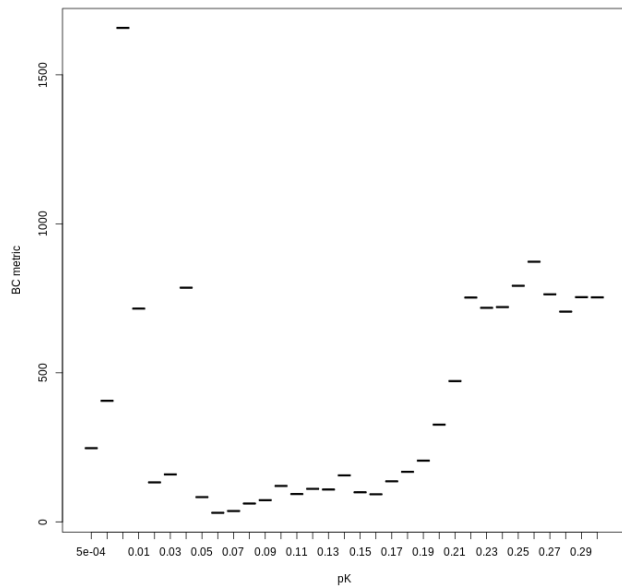
**Removing the doublets.** Next step in preprocessing is finding and removing doublets, which are barcodes where more than one cell ends up in a droplet. Overloading of cells is a common problem in scRNA-seq protocols in droplet-based methods. In a typical 10x experiment proportion of doublets is linearly dependent on the amount of loaded cells. Our data comes from an experiment using the Chromium system targeting 5000 cells. So the expected number of doublets is 682 cells according to the Chromium user guide Figure 6.

We used the DoubletFinder R package. As we don't have prior knowledge which  $pK$  value to choose, thus we use paramSweep\_v3 function with 28 first PCs to estimate the best  $pK$  value using BC metric (Figure 7). The  $pK$  value with the highest BS score is 0.005. We run doubletFinder\_v3 function with 28 first PCs,  $pN = 0.25$ ,  $pK = 0.005$ ,  $nExp = 682$ . The results can be seen on Figure 8.

We can also observe (Figure 9) that predicted doublets have more detected genes than a singlets, which is to be expected. Often only top 10 PCs are used, however with our high dimensional data results after only using 10 PCs ( $pK$  is then estimated as 0.17). Then doublets are found mostly in one neighborhood (coincidentally corresponding to cancer cells and the same few samples), which is less likely compared to results when using 28 PCs when doublets are found in many clusters, coming from many samples Figure 10.

After removing doublets we are left with 27366 cells.

**Influence of sample number on variance.** After analyzing PCA, UMAP and tSNE plots we can observe that the variance observed in the dataset might be explained by sample number. As each sample corresponds to certain individual with certain sex and age as well as to organism part and sampling site we can also note some grouping when colouring by each of these features. In order to minimize the effect that samples have on variance in the data we scale the data and regress sample variable against each gene/feature. That



**Fig. 7.** BC metric score of pK estimations done by paramSweep\_v3 function from DoubletFinder

hopefully will allow us to get more accurate clustering results.

**Unsupervised clustering.** We conducted unsupervised clustering on data scaled with regressing out for samples and without correction both times using Luovain algorithm implemented in FindClusters() function.

Resolution 0.6, 0.8, 1, 1.2 were explored for deciding on optimal clustering both before (Figure 15) and after (Figure 17) correction. Furthermore we checked how cluster cells were distributed between clusters depending on resolution before (16) and after (Figure 18) correction.

Finally have set the resolution parameter to 0.6 in both cases.

When clustering the data not corrected for the sample the algorithm found 26 clusters (plot and plot) and after correction 44 clusters.

Furthermore we searched for cluster biomarkers: differentially expressed features in different clusters. We used FindAllMarkers function to find positive markers for every cluster compared to all remaining cells. We chose to only test genes that are expressed in 0.1 cells in either of the two populations and ones that show on average, at least X-fold difference (log-scale) between the two groups of cells (logfc.threshold=0.25).

Finally we analyze cluster markers and try to make an inference about the cell types. We used both approaches first: analyze top markers by cluster and based on them try to infer cell type and second: identify clusters by searching where specific cell type markers are highly expressed.

Moreover we used the sc\_type\_score function from ScType R package which allows us to automatically assign cell identity to clusters and is compatible with Seurat package (6). The sc\_type\_score function was called only for positive cluster markers.

## Results

**Correcting possible batch effects.** After analyzing PCA, tSNE and UMAP plots we can easily notice that cells with the same metadata variable are often grouped together. However here only information of possible batches we have are samples which don't include information about possible differences coming from the experiment environment. Even though we may be eager to correct data by regressing out for each metadata variable it may not necessarily be useful in this case. When studying cancer it has been noted that variables such as sex and age might influence survival rate of certain types of cancer (7). Thus if we regress out those variables it may obscure important biological variables that could be useful during making inference. Similarly other metadata variables like sampling site and organism part actually bring important information in understanding the variance in data. In conclusion the only variable that we decided to make correction for is the sample number as it indirectly encapsulates access variation possibly brought by metadata variables. However this correction would still preserve enough differences between populations grouped by metadata variables that making inference based on them would be informative. This can be observed on plots before and after correction coloured by sex, age and organism part.

To summarize, in the situation where we don't have the obvious batches to correct for like different protocols or equipment, correcting for metadata variables depends on the type of inference we plan to make and the biological process itself.

**Assigning identity to clusters.** While analyzing clustering results we used two different approaches. First one is to analyze top markers by the cluster and try to infer cell type that way. Second approach is to try to identify cluster types by searching where specific cell type markers are highly expressed. In this approach really useful is Human Protein Atlas (8)<sup>2</sup>.

**Clustering before correction.** For each cluster we identified markers that differentiate it from other clusters. Most of the time analysis of most significant markers for each cluster should give us some idea what kind of cell we should expect in the cluster. Top ten most significant markers for each clusters can be seen in the Figure 27.

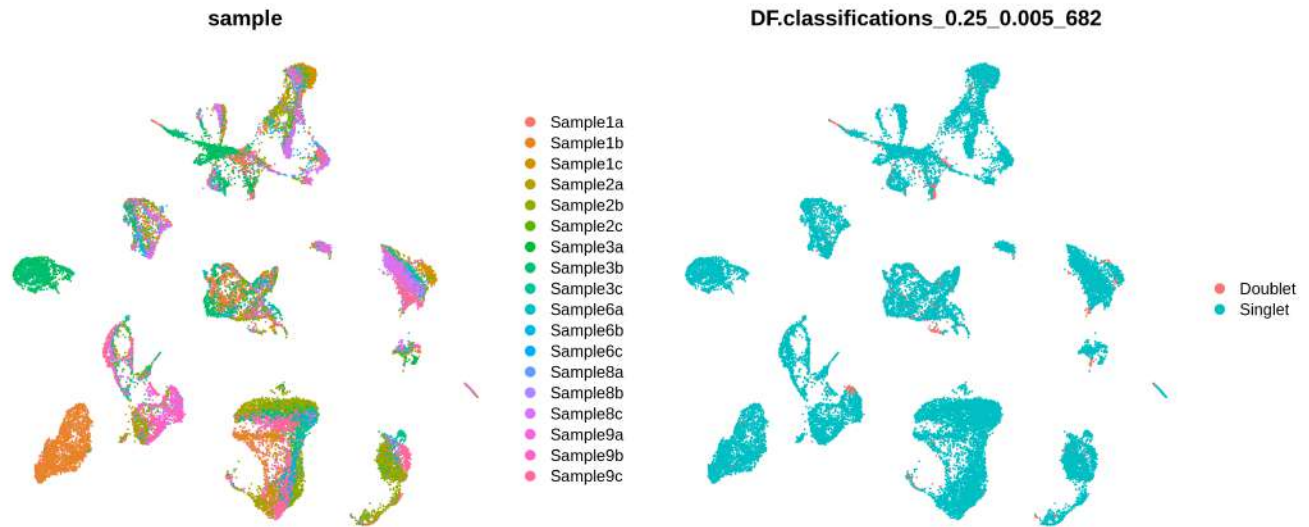
All clusters that we were able to assign cell identities to can be seen in Table 1.

**Identity by cell type markers.** The NK cell markers: NKG7 (ENSG00000105374), GNLY (ENSG00000115523) were highly expressed in cluster 3 as seen on Figure 28. Differential cluster 3 markers included CCL4 and CCL5 which are major HIV-suppressive factors produced by CD8+ T-cells. That would suggest that cluster 3 is rather not homogeneous.

Along with NK cell markers cluster 3 is characterized by the expression of  $\gamma\delta$  T cells: TRDC

<sup>2</sup><https://www.proteinatlas.org>





**Fig. 8.** Distribution of doublets between samples,  $pK = 0.005$

Cell type	Clusters
NK cells	3
$\gamma\delta$ T	3
CD8+ T	3
Naive CD4+ T	0, 25
Memory CD4+ T	15
CD14+ Monocytes	9
CD16+ Monocytes	7
Mast cells	23
B cells	4, 18
Neuronal cells	19
Fibroblasts	8, 10, 12, 21
IgA+ plasma cell	1
IgG+ memory B cells	20
Smooth muscle cell	24

**Table 1.** Suggested cluster identity by high expression of cell type markers

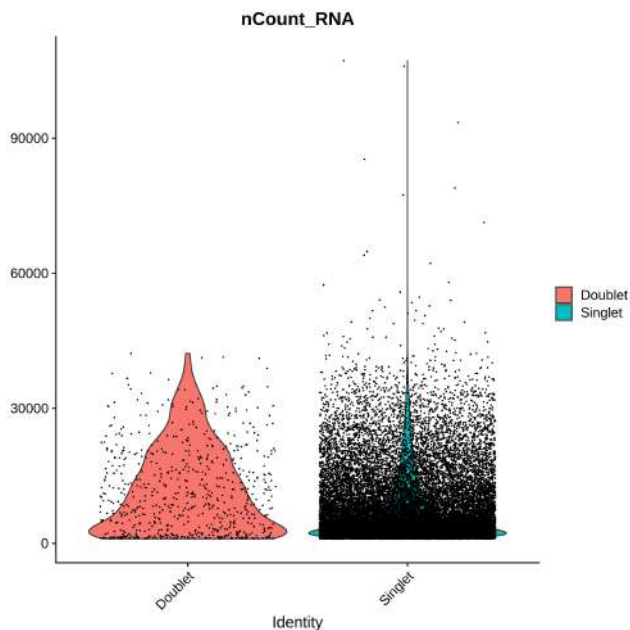
(ENSG00000211829), TRGC2 (ENSG00000227191) and TRGC1 (ENSG00000211689) (29).

Another cell population present in cluster 3 are CD8+ T cells, which were identified by their markers: CD8A (ENSG00000153563), CD8B (ENSG00000172116), which can be seen on Figure 30.

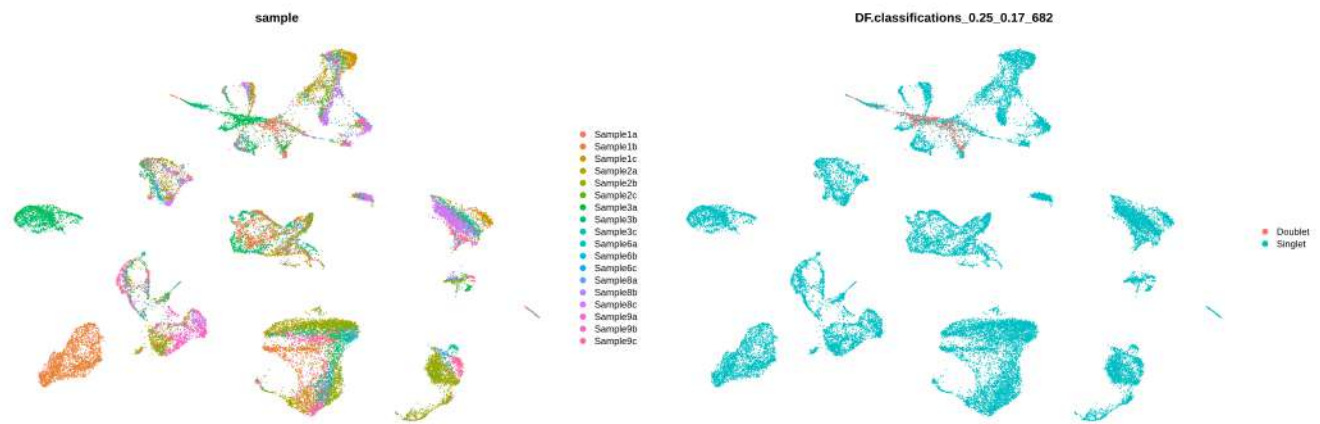
The CD4+ T cells markers: CD4 (ENSG0000010610), CCR7 (ENSG00000126353), IL7R (ENSG00000168685) allowed us to identify naive CD4+ T (CCR7+) population in clusters 0, 25 and memory CD4+ T (CCR7-) population in cluster 15 (Figure 31).

Another cell population identified by its canonical markers: MS4A1 (ENSG00000156738), CD19 (ENSG00000177455) were B cells found in clusters 4 and 18 as seen on 32.

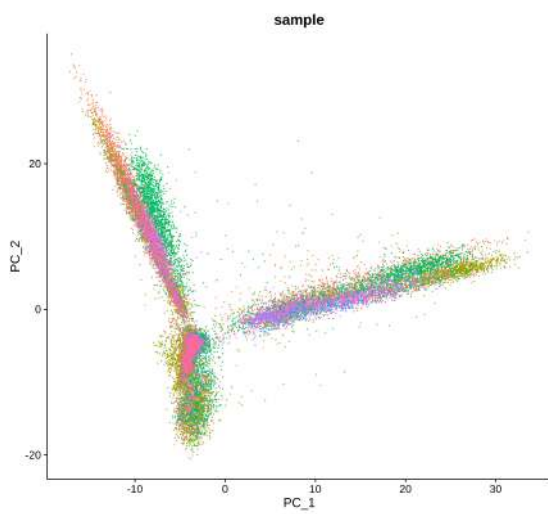
Mast cells are a type of granulocyte that is part of immune and neuroimmune systems. After analysis of mast cell markers such as The mast cell markers such as TPSAB1 (ENSG00000172236), TPSB2 (ENSG00000197253), GATA1 (ENSG00000102145),



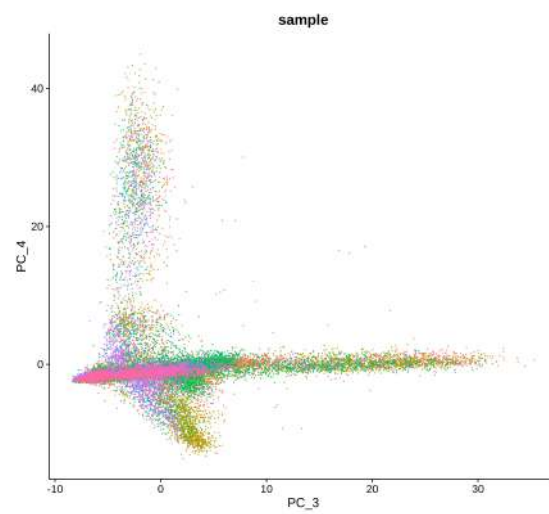
**Fig. 9.** Number of UMI counts in doublets and singlets,  $pK = 0.005$



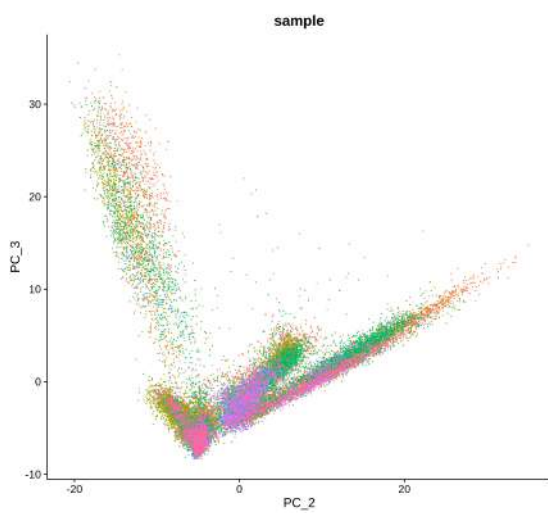
**Fig. 10.** Distribution of doublets between samples,  $pK = 0.017$



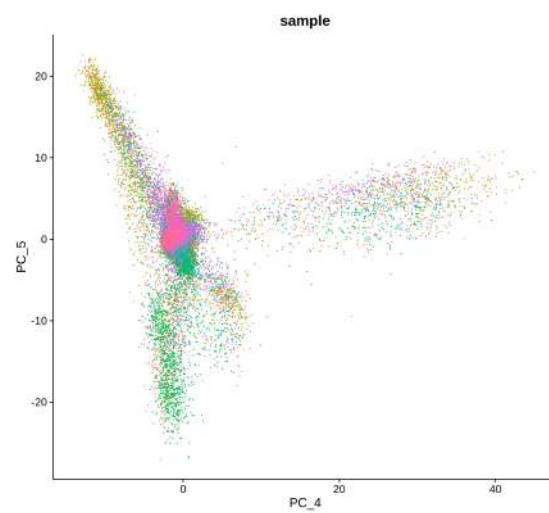
**Fig. 11.** PC1 and PC2 by sample before correction



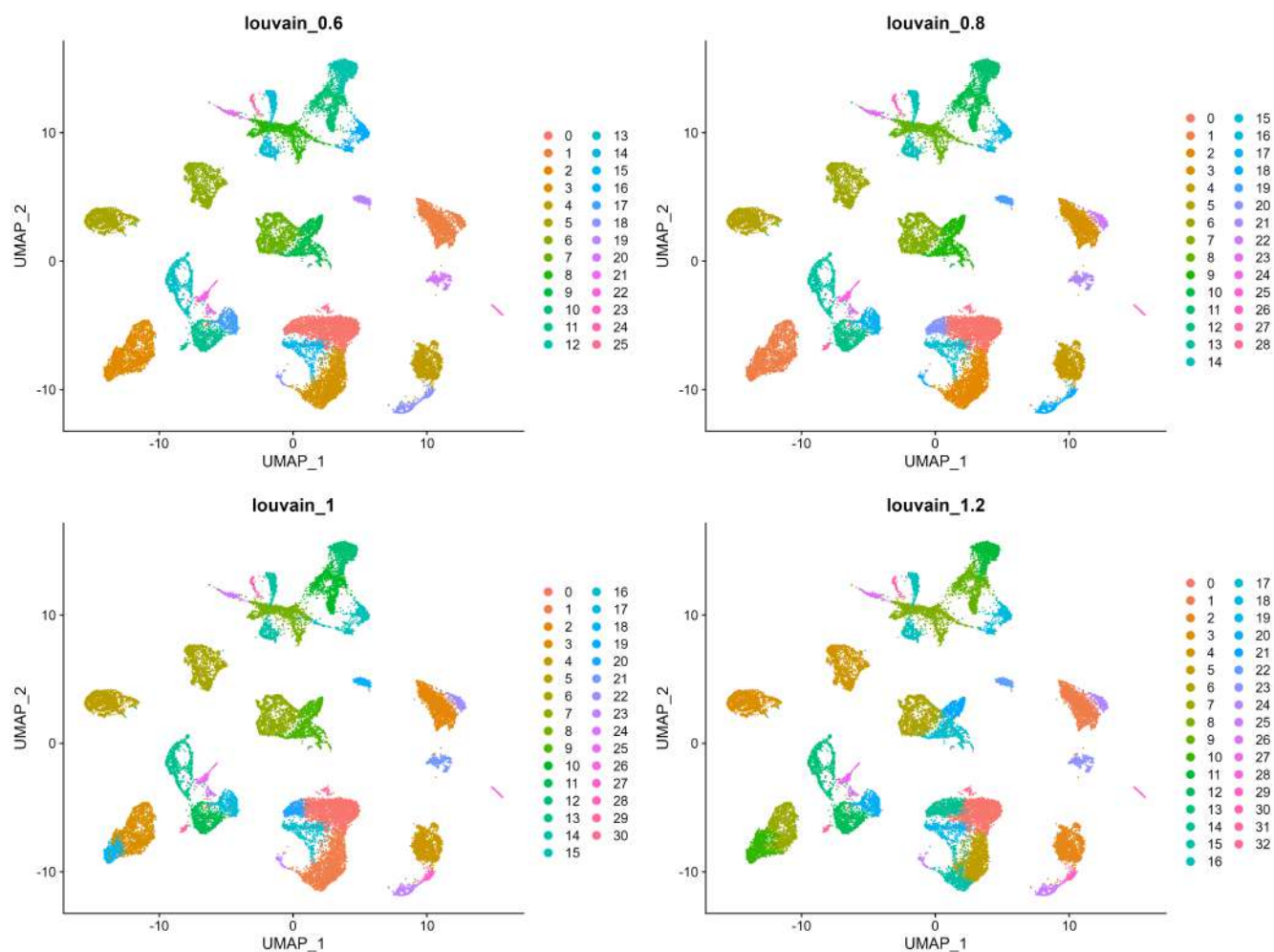
**Fig. 13.** PC3 and PC4 by sample before correction



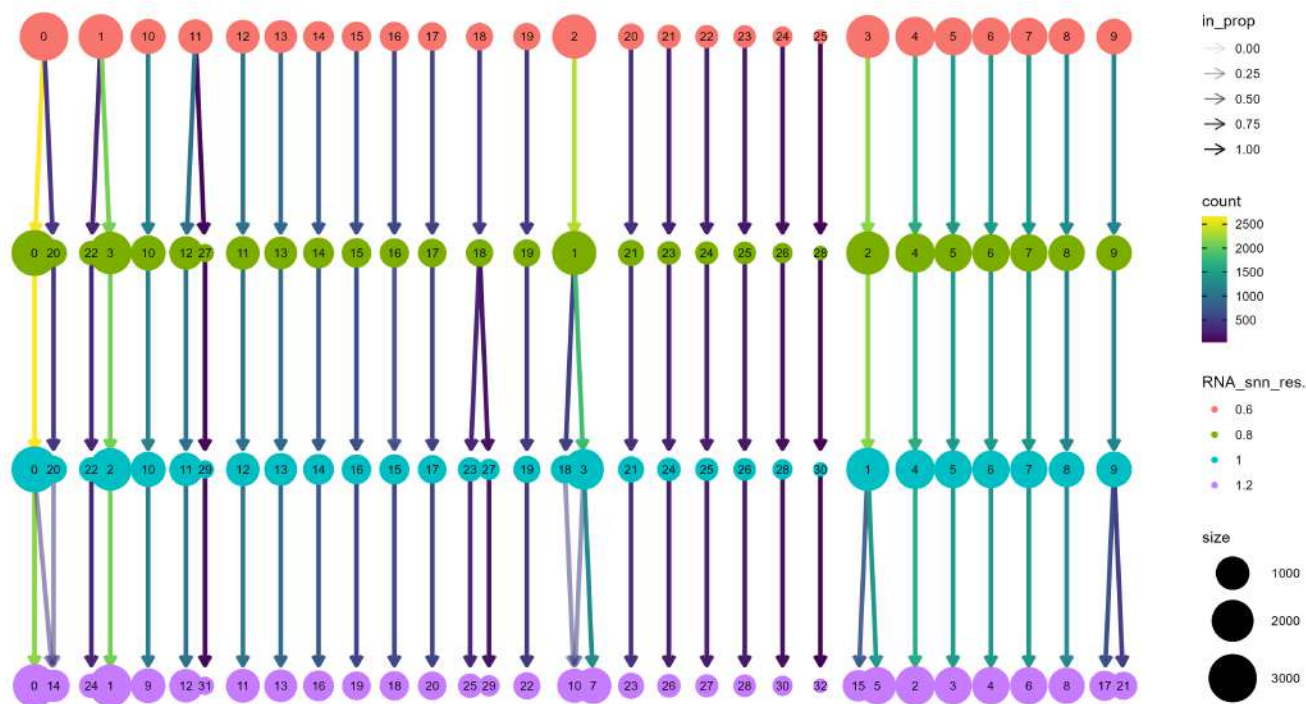
**Fig. 12.** PC2 and PC3 by sample before correction



**Fig. 14.** PC4 and PC5 by sample before correction

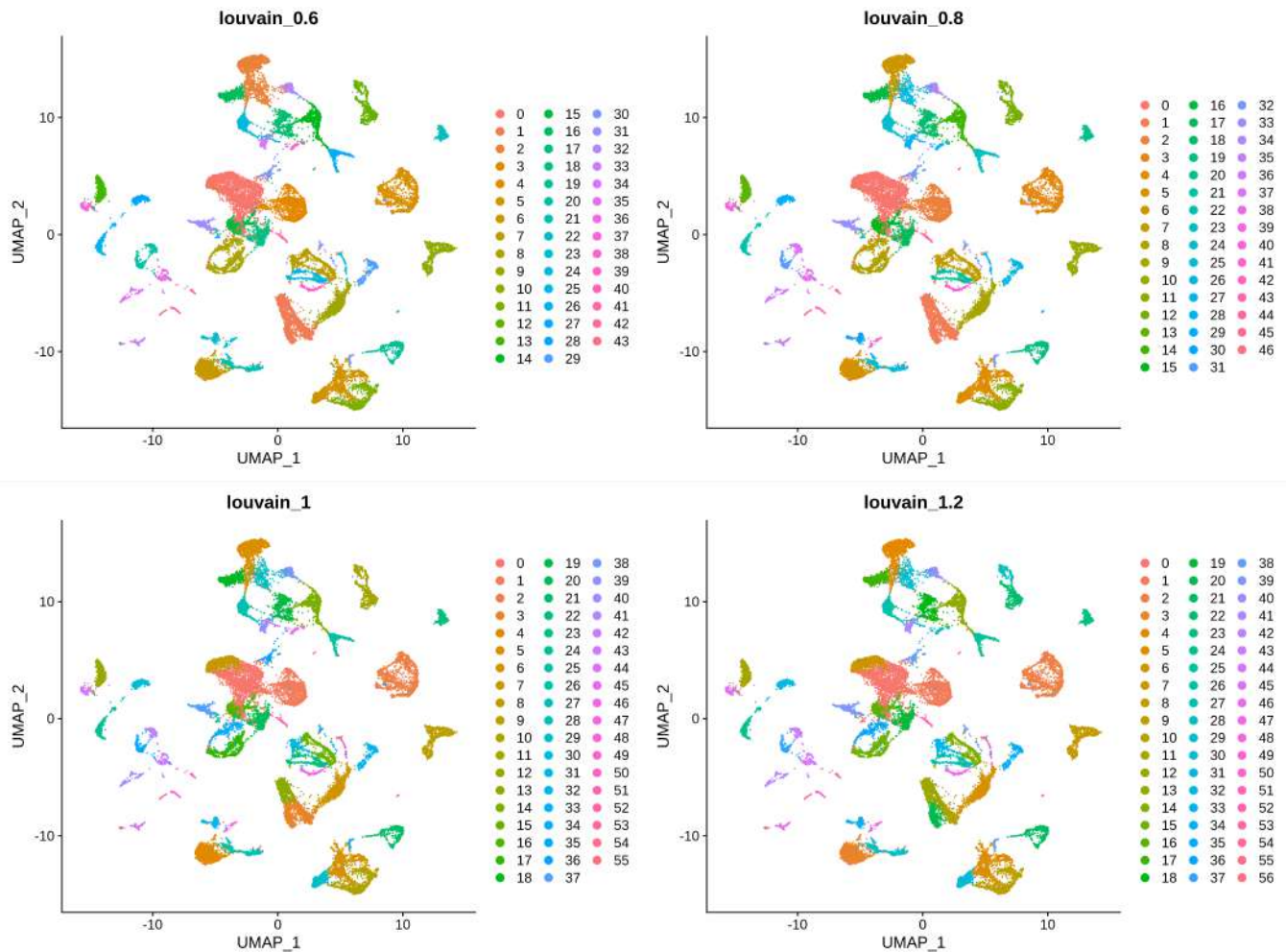


**Fig. 15.** Clustering results depending on resolution before correction



**Fig. 16.** Distribution of cells between clusters depending on resolution before correction





**Fig. 17.** Clustering results depending on resolution after correction

GATA2 (ENSG00000179348) we identified cluster 23 as most likely to be predominantly grouping mast cells (33).

When searching for monocytes clusters we used markers that can identify CD14+ monocytes: CD14 (ENSG00000170458) and CD16+ monocytes: FCGR3A (ENSG00000203747) as well as general monocytes markers: ITGAM (ENSG00000169896) and LYZ (ENSG00000090382) (9). Both had the highest levels of expression in clusters 7 and 9. However we were unable to confidently differentiate between these two types of monocytes. Slight difference in expression levels might suggest that cluster 7 consists mostly of CD16+ monocytes while cluster 9 mostly of CD14+ monocytes as seen on Figure 34.

Furthermore we search for markers that can help us differentiate between cluster 7 and 9 and top two ones were: S100A9 (ENSG00000163220) and S100A8 (ENSG00000143546) which are known monocyte markers and their expression levels were higher in cluster 7. Higher mRNA levels of S100A9 were previously found in CD16+ monocytes (10), (11) and is seen in cluster 7 in our data Figure 35. This brings us to the following identity assignment: cluster 7 - CD16+ monocytes and cluster 9 - CD14+ monocytes.

Another set of markers present in the cells are smooth muscle

markers such as: ACTG2 (ENSG00000163017), TAGLN (ENSG00000149591), MYH11 (ENSG00000133392), CNN1 (ENSG00000130176), DES (ENSG00000175084), SYNPO2 (ENSG00000172403) Figure 36.

Neuronal cell marker NCAM1 had highest levels of expression in cluster 19 (Figure 37).

Other clusters that were identified by checking expression level of cell type markers were fibroblasts. To identify fibroblast clusters we use positive markers: THY1 (ENSG00000154096), COL1A1 (ENSG00000108821), COL3A1 (ENSG00000168542), COL1A2 (ENSG00000164692), COL5A1 (ENSG00000130635) (Figure 38) and LUM (ENSG00000139329), DCN (ENSG0000011465), LOXL1 (ENSG00000129038), FBLN1 (ENSG00000077942), FBLN2 (ENSG00000163520) (Figure 39) and negative marker RGS5 (ENSG00000143248) which is not expressed in fibroblasts (12).

In this case we can observe that clusters identified as fibroblasts can be divided into the ones consisting mostly of normal cells and those consisting mostly of cancer cells, which is visualized on Figure 40 and Figure 41.

To summarize in normal cells most we can find highly ex-



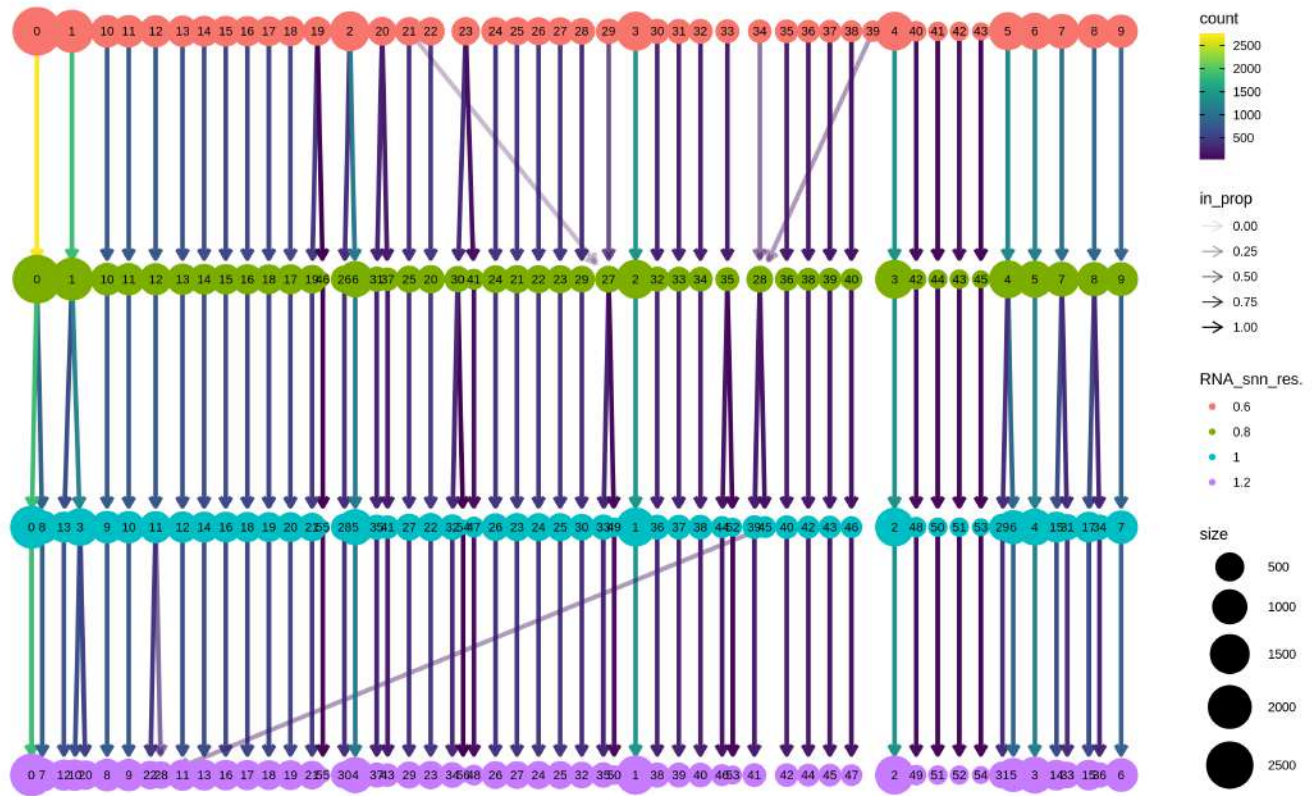


Fig. 18. Distribution of cells between clusters depending on resolution after correction

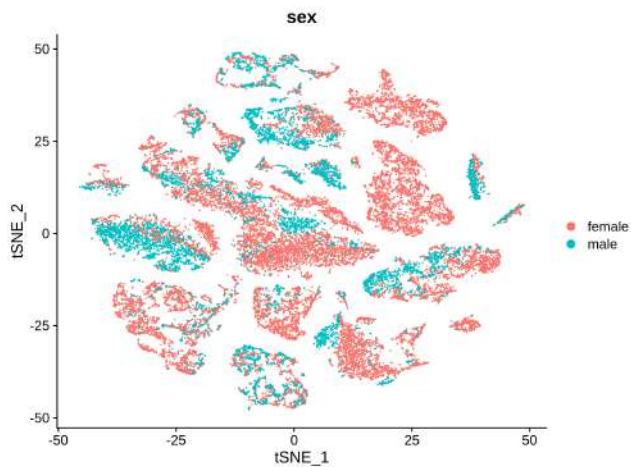


Fig. 19. Cell population by sex before correction for sample number

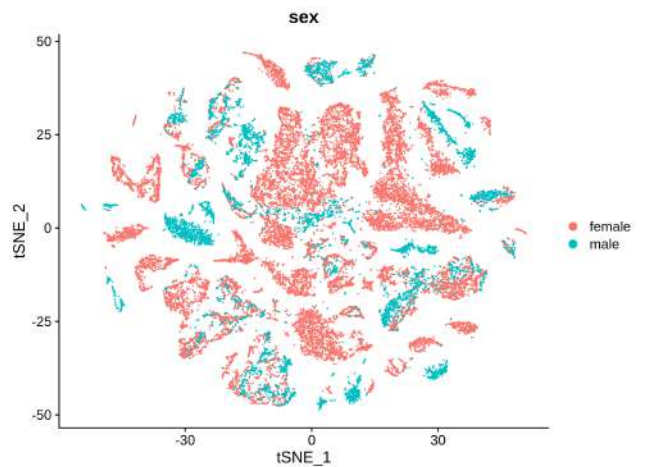
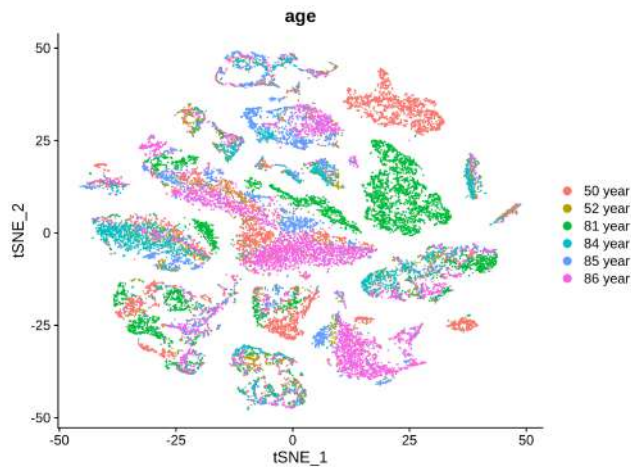
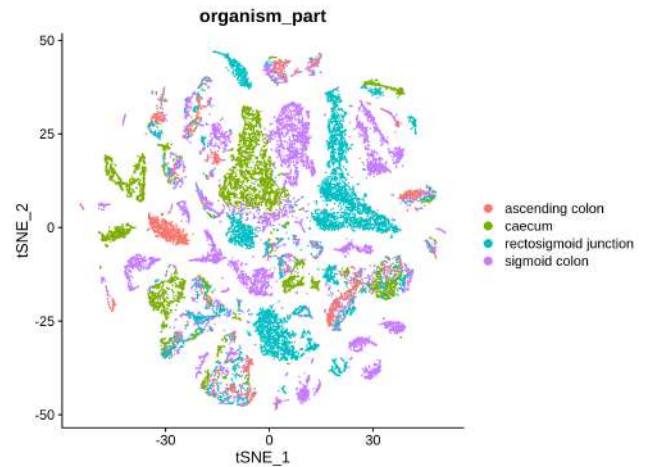


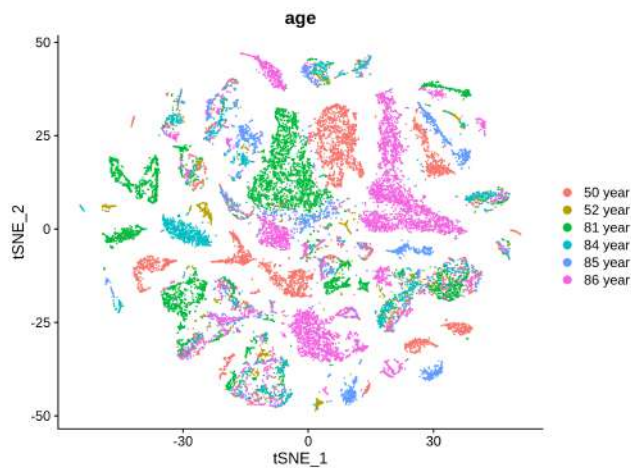
Fig. 20. Cell population by sex after correction for sample number



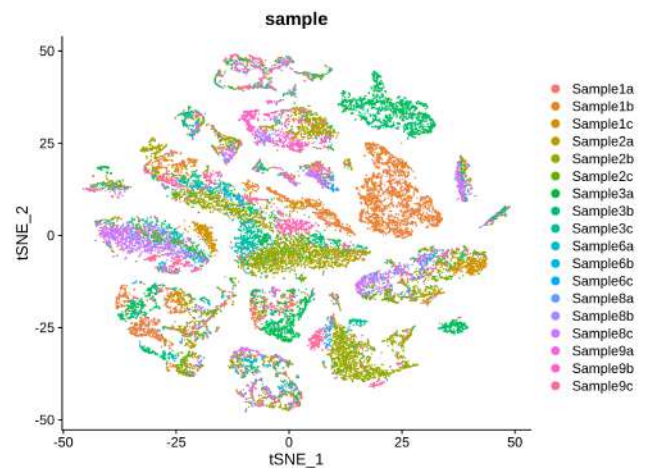
**Fig. 21.** Cell population by age before correction for sample number



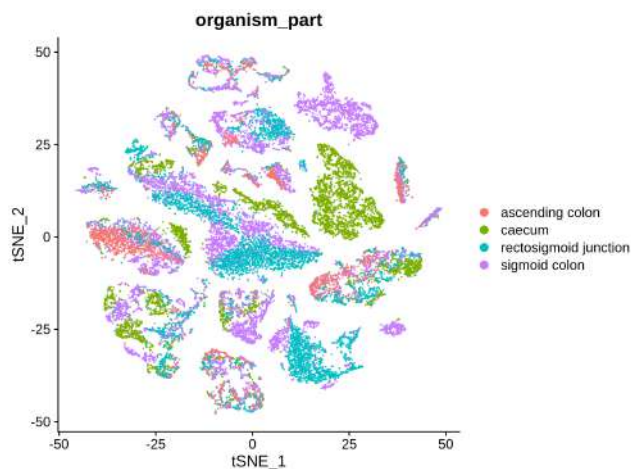
**Fig. 24.** Cell population by organism part from which sample was collected after correction for sample number



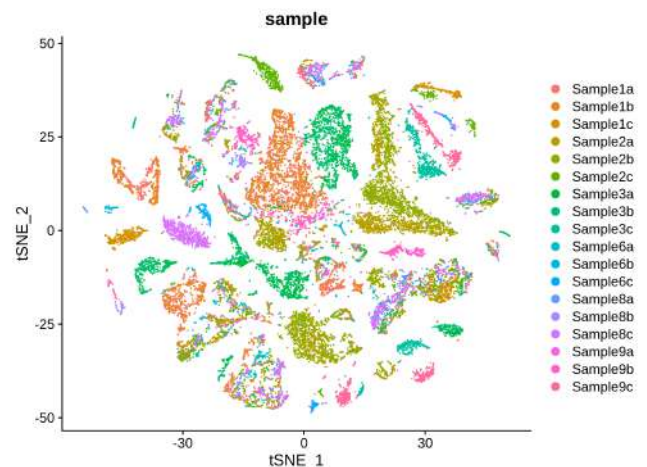
**Fig. 22.** Cell population by age after correction for sample number



**Fig. 25.** Cell population by sample before correction for sample number



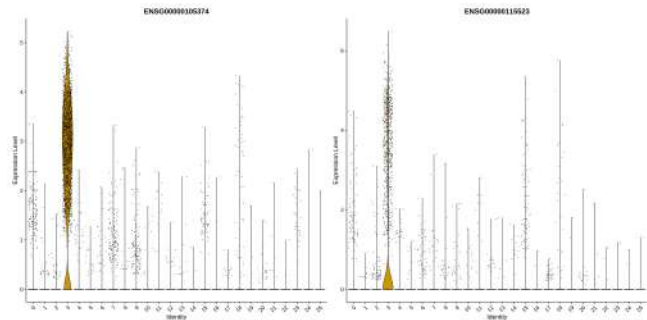
**Fig. 23.** Cell population by organism part from which sample was collected before correction for sample number



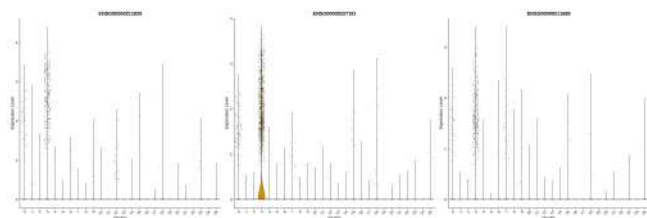
**Fig. 26.** Cell population by sample after correction for sample number

	1	2	3	4	5	6	7	8	9	10
Cluster 0	IL7R	TRAC	KLRB1	CD3D	LTB	LTB	CD2	TRBC1	TRBC1	CD3E
Cluster 1	IGHA1	IGHA2	JCHAIN	IGKC	IGLC2	IGLL5	MZB1	IGHA2	SSR4	TNFRSF17
Cluster 2	REG4	KRT18	AGR2	S100P	KRT8	KRT19	EPCAM	IFIT2	TSPAN8	TN
Cluster 3	NG2	GNLY	GZMA	CCLA	CCLA	GZMK	CCL5	CCL5	CCL4	GZMB
Cluster 4	IGHM	CD79A	MS4A1	VPREB3	CD37	CD74	CD79B	HLA-DRA	HLA-DRA	HLA-DPB1
Cluster 5	MMP7	DEFB1	DEFB1	PSCA	LCN2	C19orf33	CEACAM6	KRT19	KRT16	TMSF4
Cluster 6	CLDN5	PLVAP	RAMP2	VWF	GNG11	ACKR1	OCL14	FABP4	CAV1	FABP5
Cluster 7	SPP1	S100A9	CXCL5	S100A8	APOC1	IFI30	FTL	TYROBP	FCER1G	LYZ
Cluster 8	COL1A1	COL1A2	COL3A1	CTHRC1	FN1	BGN	COMP	POSTN	SPARC	TAGLN
Cluster 9	C1QB	C1QA	C1QC	HLA-DPA1	HLA-DRB1	HLA-DRA	HLA-DRA	HLA-DPB1	MS4A6A	HLA-DRB3
Cluster 10	DPT	MGP	C7	APOD	GSN	DCN	OCL11	ADH1B	CCDC80	FBLN1
Cluster 11	CD24	SPINK1	PRAC1	FABP1	GPX2	TFF3	RPL12	RPS6	RPL31	RPS18
Cluster 12	CFD	CFD	IGFBP6	CLEC3B	DCN	MFAP5	SFRP2	MGP	PLAC9	EFEMP1
Cluster 13	FABP1	CA1	PHGR1	CKB	MT1G	PIGR	CA2	LGALS4	C15orf48	FXYD3
Cluster 14	ACTA2	TAGLN	RGSS	ADIRF	MYL9	MUSTN1	TPM2	PLN	NDUFA4L2	CALD1
Cluster 15	HLA-A	CD3D	TRAC	CD7	HLA-B	HLA-B	HLA-B	BATF	CD2	RPS9
Cluster 16	ADAMDEC1	CXCL14	CCL13	CCL8	CEBPD	APOE	ABCA8	CFH	MFAP4	LTBP4
Cluster 17	SPINK1	CEACAM5	TFF3	PIGR	LCN2	FAM3D	CD24	PLCB4	ATP1B1	LEFTY1
Cluster 18	MS4A1	TCL1A	CD79B	CD79A	VPREB3	IRAG2	RG813	MEF2B	IRF8	PTTG1
Cluster 19	CRYAB	S100B	GPM6B	CLU	FXYD1	PLP1	LG14	NRXN1	ALDH1A1	PMP22
Cluster 20	IGHG1	IGHG3	IGHG3	IGHG4	IGHG5	IGHG5	MZB1	DERL3	JSRP1	DERL3
Cluster 21	CCN1	SERPINE1	IGF1	CTHRC1	BGN	C3	PTGDS	COL5A1	CCDC80	SGK1
Cluster 22	ZG16	SPINK4	FCGBP	MUC2	ITLN1	MUC2	FCGBP	WDFC2	CLCA1	KLK1
Cluster 23	TPSB2	TPSAB1	CPA3	CTSG	HPGD5	MS4A2	HPGD	LTC4S	VWASA	SLC18A2
Cluster 24	ACTG2	DES	CNN1	MYH11	MYH11	PLN	PCP4	SYNPO2	LMOD1	SYNM
Cluster 25	GABBR1	GABBR1	GABBR1	GABBR1	UBD	UBD	GABBR1	GABBR1	GABBR1	CD2

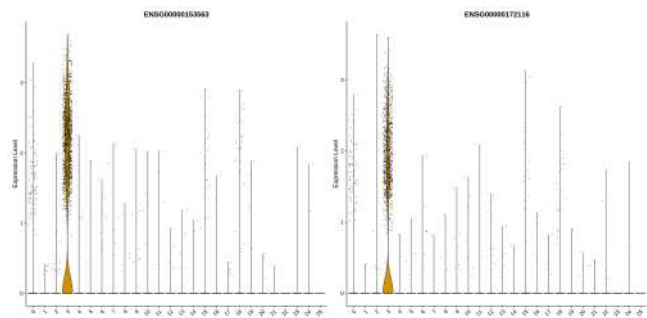
**Fig. 27.** Top 10 significant markers for clusters before correction



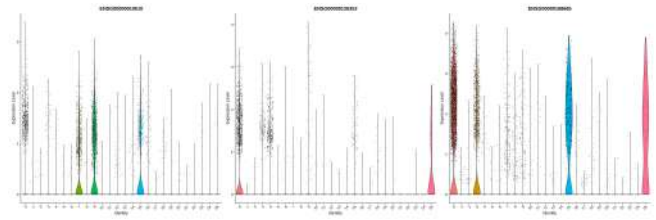
**Fig. 28.** NK cell markers in clusters before correction  
NKG7 (ENSG00000105374), GNLY (ENSG00000115523)



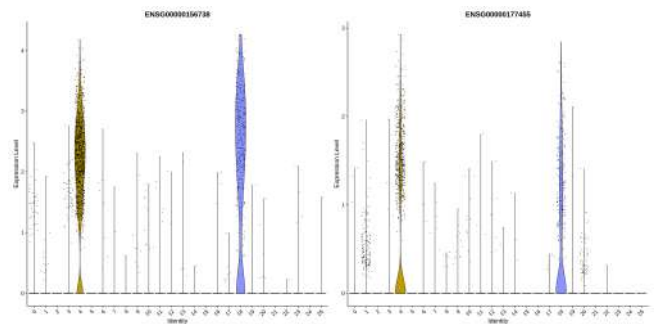
**Fig. 29.**  $\gamma\delta$  T cell markers in clusters before correction  
TRDC (ENSG00000211829), TRGC2 (ENSG00000227191) and TRGC1 (ENSG00000211689)



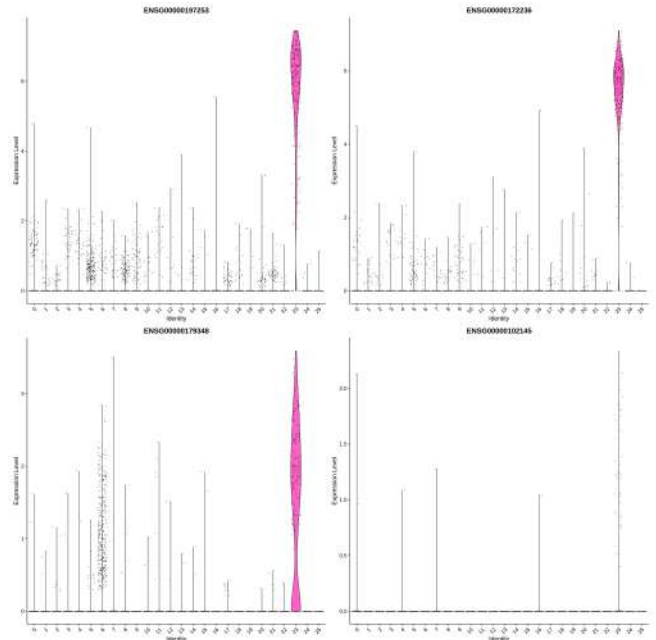
**Fig. 30.** CD8+ T cell markers in clusters before correction  
CD8A (ENSG00000153563), CD8B (ENSG00000172116)



**Fig. 31.** CD4+ T cell markers in clusters before correction  
CD4 (ENSG00000010610), CCR7 (ENSG00000126353), IL7R (ENSG00000168685)

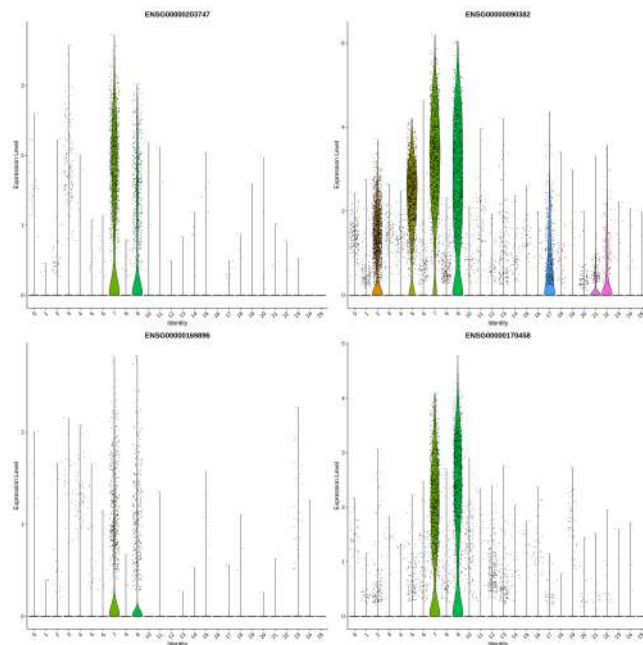


**Fig. 32.** B cell markers in clusters before correction  
MS4A1 (ENSG00000156738) and CD19 (ENSG00000177455)

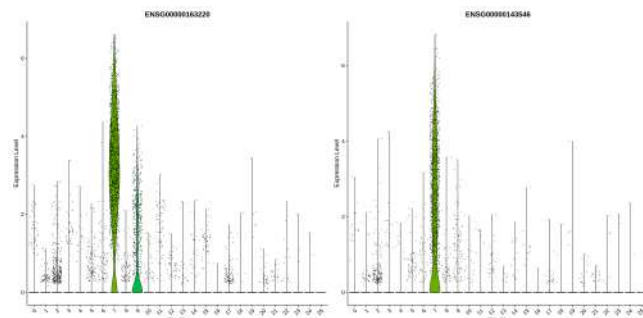


**Fig. 33.** Mast cell markers in clusters before correction  
From top left to bottom right: TPSB2 (ENSG00000197253), TPSAB1 (ENSG00000172236), GATA2 (ENSG00000179348), GATA1 (ENSG00000102145)

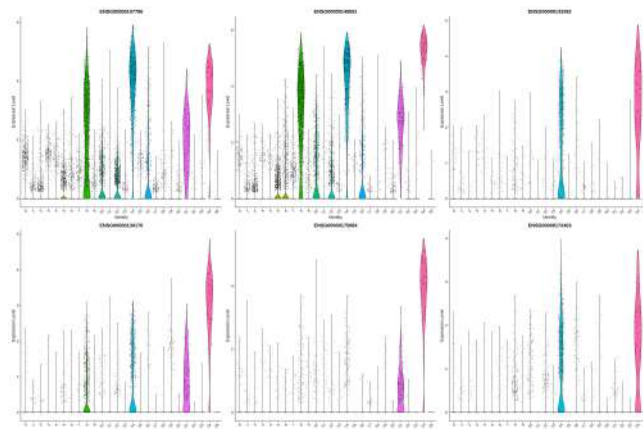




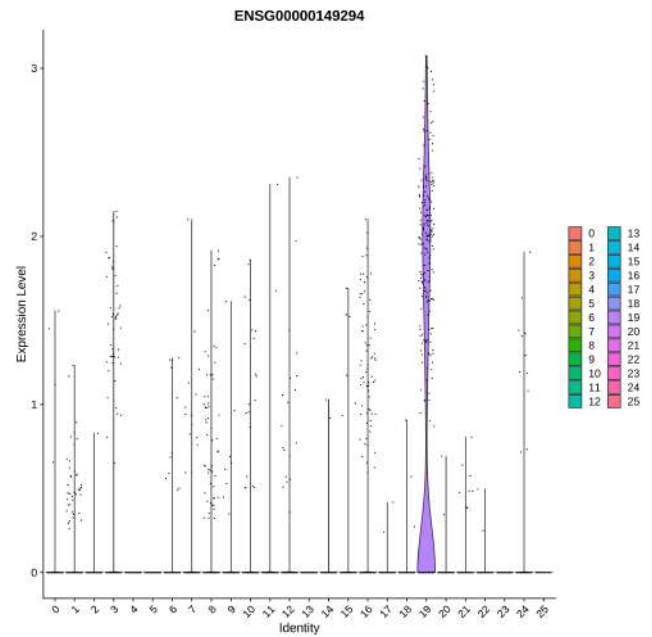
**Fig. 34.** Monocytes markers in clusters before correction  
From top left to bottom right: FCGR3A (ENSG00000203747), LYZ (ENSG00000090382), ITGAM (ENSG00000169896), CD14 (ENSG00000170458)



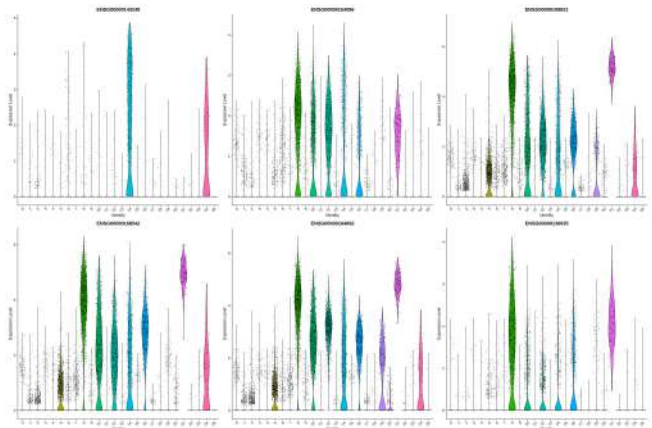
**Fig. 35.** Monocytes markers differentiating 7th and 9th cluster before correction  
S100A9 (ENSG00000163220) and S100A8 (ENSG00000143546)



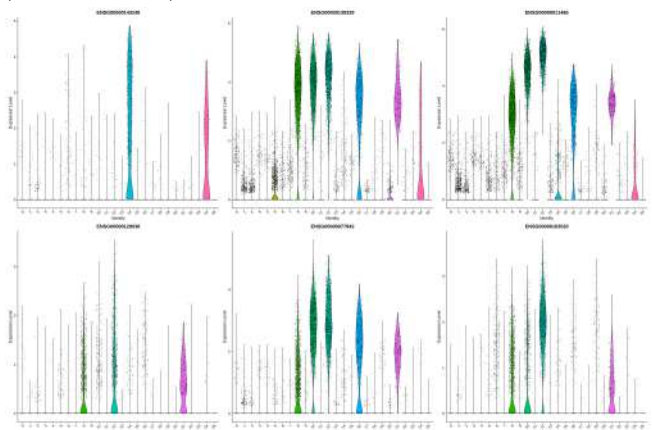
**Fig. 36.** Smooth muscle cells markers in clusters before correction  
From top left to bottom right: ACTG2 (ENSG00000163017), TAGLN (ENSG00000149591), MYH11 (ENSG00000133392), CNN1 (ENSG00000130176), DES (ENSG00000175084), SYNPO2 (ENSG00000172403)



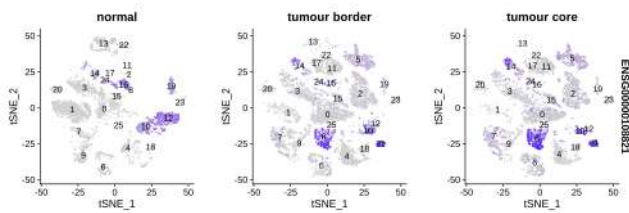
**Fig. 37.** Neuronal cell marker NCAM1 (ENSG00000149294) in clusters before correction



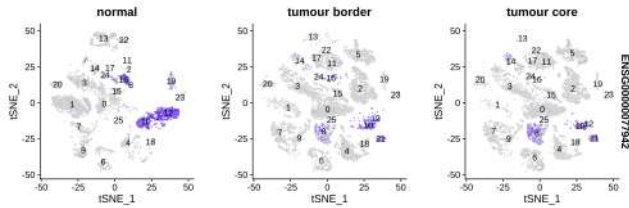
**Fig. 38.** Fibroblasts markers part 1 in clusters before correction  
From top left to bottom right: RGS5 (ENSG00000143248), THY1 (ENSG00000154096), COL1A1 (ENSG00000108821), COL3A1 (ENSG00000168542), COL1A2 (ENSG00000164692), COL5A1 (ENSG00000130635)



**Fig. 39.** Fibroblasts markers part 2 in clusters before correction  
From top left to bottom right: RGS5 (ENSG00000143248), LUM (ENSG00000139329), DCN (ENSG0000011465), LOXL1 (ENSG00000129038), FBLN1 (ENSG00000077942), FBLN2 (ENSG00000163520)



**Fig. 40.** Fibroblasts marker: COL1A1 in clusters grouped by tissue type, before correction



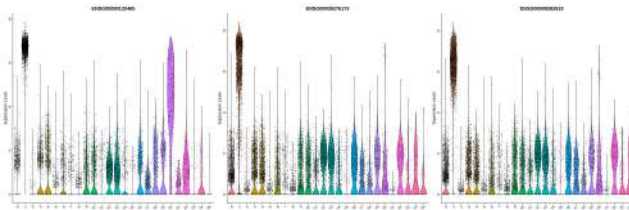
**Fig. 41.** Fibroblasts marker: FBLN1 in clusters grouped by tissue type, before correction

pressed fibroblast markers in clusters 8 and 21, while in cancer cell higher markers expression is present in clusters 10 and 12.

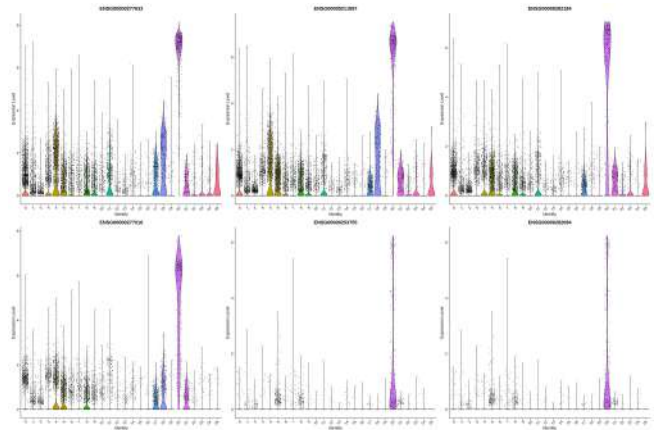
**Identity by cluster markers.** Cluster 1 is characterized by high expression of IgA+ cells markers such as IGHA1 (ENSG00000282633), IGHA2 (ENSG00000276173) and JCHAIN (ENSG00000132465), which can be seen on [Figure 42](#).

Another cluster identity we were able to assign was to cluster 20, which was characterized by high mRNA content of IgG+ cell markers such as IGHG1 (ENSG00000277633), IGHG3 (ENSG00000211897, ENSG00000282184), IGHG4 (ENSG00000277016), IGHGP (ENSG00000253755, ENSG00000282094) [Figure 43](#). Those markers are most often expressed in IgG+ memory B cells as well as plasma cells (tiller 2007). Other than in cluster 20, high expression levels of IgG+ cell markers were expressed at significant levels in cluster 18, which has been previously identified as B cell cluster. Combining this information suggests that cluster 18 probably contains many different types of B cells.

**Comparison with ScType results.** Comparing our manual classification of clusters to ScType classification we can see clusters that in both cases had the same identification were 0, 15 as naive CD4+ T cells, 7 as CD16+ monocytes/non-classical monocytes. Cluster 3 was classified as CD8+ T and NK cells, which is consistent with our classification as CD8+



**Fig. 42.** IgA+ plasma cell markers in cluster 1 before correction  
JCHAIN (ENSG00000132465), IGHA2 (ENSG00000276173), IGHA1 (ENSG00000282633)



**Fig. 43.** IgG+ plasma cell markers in cluster 20 before correction  
IGHG1 (ENSG00000277633), IGHG3 (ENSG00000211897, ENSG00000282184), IGHG4 (ENSG00000277016), IGHGP (ENSG00000253755, ENSG00000282094)

Cell type	Clusters
NK cells	9, 10, 25, 38
$\gamma\delta$ T	9, 25, 38
CD8+ T	9, 38
Naive CD4+ T	1
Memory CD4+ T	5, 10, 11, 19
Monocytes	5, 11, 19
Mast cells	33
B cells	6, 21, 23
Neuronal cells	22
Fibroblasts	2, 14, 32, 34
IgA+ plasma cell	13
Smooth muscle cell	12, 32, 39

**Table 2.** Suggested cluster identity by high expression of cell type markers after correction

T, NK and  $\gamma\delta$  T cells. In our manual classification we assigned the identity of memory CD4+ T cells to cluster 15, however ScType classified it as naive CD4+ T cells, which is understandable as these cell types are difficult to differentiate and classification depends on choice of markers.

The ScType algorithm classified cluster 1 as naive B cells, which doesn't necessarily exclude presence of IgA+ plasma cells, however it might suggest that rather than plasma cells the cluster 1 population has a higher percentage of different types of IgA+ B cells. Similarly cluster 20 classified by us as IgG+ plasma cells was assigned a naive B cell type by ScType.

The comparison is shown on [Figure 44](#).

Further analysis revealed that some clusters that share cell type identity (ScType classification) consisted mostly of cells from normal tissue while the other mostly of cells from cancer tissue which is presented [Figure 45](#)

**Clustering after correction.** Again for finding out cluster identity we used top ten most significant markers for each clusters ([Figure 46](#)).

All clusters that we were able to assign cell identities to can be seen in [Table ??](#).



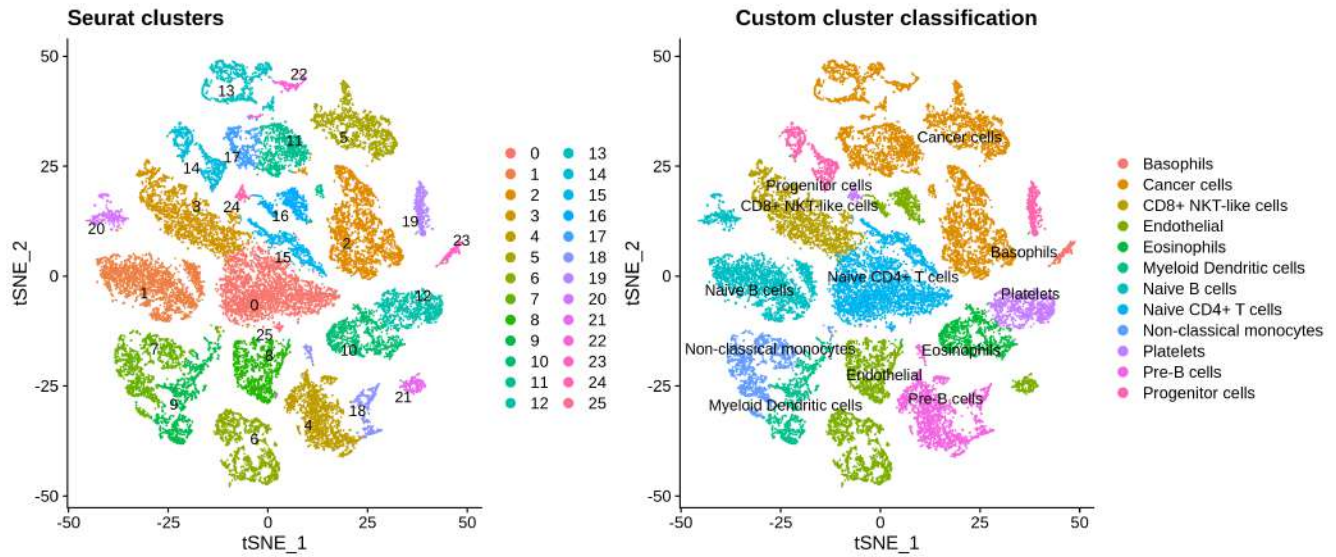


Fig. 44. Seurat clusters vs ScType cluster identities before correction

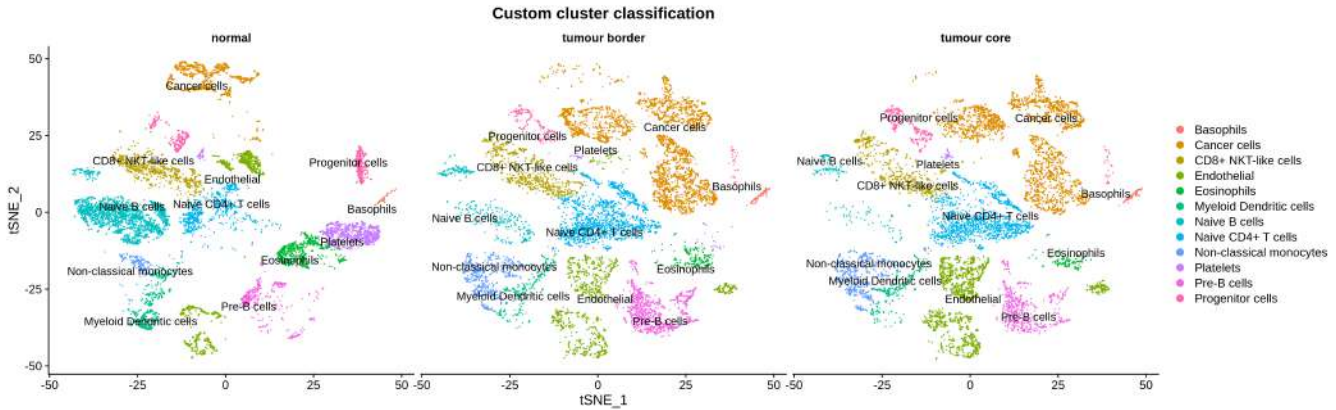


Fig. 45. ScType cluster identities by sampling site before correction

	1	2	3	4	5	6	7	8	9	10
Cluster 0	REGA	ADG2	KRT18	S100P	KRT18	EPDM	KRT18	IFIT7	TSPY8B	TIN
Cluster 1	TRAC	LTB	LTB	ILTR	BIRC3	CD30	TRBC1	TNFRSF4	BTG1	TRBC1
Cluster 2	CD13	CD13	CD13	SPR2	IGFBP5	MDP	CD13	MDP	CD13	MDP
Cluster 3	MDP	CD13	CD13	SPR2	IGFBP5	MDP	CD13	MDP	CD13	MDP
Cluster 4	CD13	CD13	CD13	SPR2	IGFBP5	MDP	CD13	MDP	CD13	MDP
Cluster 5	CD13	CD13	CD13	SPR2	IGFBP5	MDP	CD13	MDP	CD13	MDP
Cluster 6	CD13	CD13	CD13	SPR2	IGFBP5	MDP	CD13	MDP	CD13	MDP
Cluster 7	CD13	CD13	CD13	SPR2	IGFBP5	MDP	CD13	MDP	CD13	MDP
Cluster 8	CD13	CD13	CD13	SPR2	IGFBP5	MDP	CD13	MDP	CD13	MDP
Cluster 9	CD13	CD13	CD13	SPR2	IGFBP5	MDP	CD13	MDP	CD13	MDP
Cluster 10	CD13	CD13	CD13	SPR2	IGFBP5	MDP	CD13	MDP	CD13	MDP
Cluster 11	CD13	CD13	CD13	SPR2	IGFBP5	MDP	CD13	MDP	CD13	MDP
Cluster 12	CD13	CD13	CD13	SPR2	IGFBP5	MDP	CD13	MDP	CD13	MDP
Cluster 13	CD13	CD13	CD13	SPR2	IGFBP5	MDP	CD13	MDP	CD13	MDP
Cluster 14	CD13	CD13	CD13	SPR2	IGFBP5	MDP	CD13	MDP	CD13	MDP
Cluster 15	CD13	CD13	CD13	SPR2	IGFBP5	MDP	CD13	MDP	CD13	MDP
Cluster 16	CD13	CD13	CD13	SPR2	IGFBP5	MDP	CD13	MDP	CD13	MDP
Cluster 17	CD13	CD13	CD13	SPR2	IGFBP5	MDP	CD13	MDP	CD13	MDP
Cluster 18	CD13	CD13	CD13	SPR2	IGFBP5	MDP	CD13	MDP	CD13	MDP
Cluster 19	CD13	CD13	CD13	SPR2	IGFBP5	MDP	CD13	MDP	CD13	MDP
Cluster 20	CD13	CD13	CD13	SPR2	IGFBP5	MDP	CD13	MDP	CD13	MDP
Cluster 21	CD13	CD13	CD13	SPR2	IGFBP5	MDP	CD13	MDP	CD13	MDP
Cluster 22	CD13	CD13	CD13	SPR2	IGFBP5	MDP	CD13	MDP	CD13	MDP
Cluster 23	CD13	CD13	CD13	SPR2	IGFBP5	MDP	CD13	MDP	CD13	MDP
Cluster 24	CD13	CD13	CD13	SPR2	IGFBP5	MDP	CD13	MDP	CD13	MDP
Cluster 25	CD13	CD13	CD13	SPR2	IGFBP5	MDP	CD13	MDP	CD13	MDP
Cluster 26	CD13	CD13	CD13	SPR2	IGFBP5	MDP	CD13	MDP	CD13	MDP
Cluster 27	CD13	CD13	CD13	SPR2	IGFBP5	MDP	CD13	MDP	CD13	MDP
Cluster 28	CD13	CD13	CD13	SPR2	IGFBP5	MDP	CD13	MDP	CD13	MDP
Cluster 29	CD13	CD13	CD13	SPR2	IGFBP5	MDP	CD13	MDP	CD13	MDP
Cluster 30	CD13	CD13	CD13	SPR2	IGFBP5	MDP	CD13	MDP	CD13	MDP
Cluster 31	CD13	CD13	CD13	SPR2	IGFBP5	MDP	CD13	MDP	CD13	MDP
Cluster 32	CD13	CD13	CD13	SPR2	IGFBP5	MDP	CD13	MDP	CD13	MDP
Cluster 33	CD13	CD13	CD13	SPR2	IGFBP5	MDP	CD13	MDP	CD13	MDP
Cluster 34	CD13	CD13	CD13	SPR2	IGFBP5	MDP	CD13	MDP	CD13	MDP
Cluster 35	CD13	CD13	CD13	SPR2	IGFBP5	MDP	CD13	MDP	CD13	MDP
Cluster 36	CD13	CD13	CD13	SPR2	IGFBP5	MDP	CD13	MDP	CD13	MDP
Cluster 37	CD13	CD13	CD13	SPR2	IGFBP5	MDP	CD13	MDP	CD13	MDP
Cluster 38	CD13	CD13	CD13	SPR2	IGFBP5	MDP	CD13	MDP	CD13	MDP
Cluster 39	CD13	CD13	CD13	SPR2	IGFBP5	MDP	CD13	MDP	CD13	MDP
Cluster 40	CD13	CD13	CD13	SPR2	IGFBP5	MDP	CD13	MDP	CD13	MDP
Cluster 41	CD13	CD13	CD13	SPR2	IGFBP5	MDP	CD13	MDP	CD13	MDP
Cluster 42	CD13	CD13	CD13	SPR2	IGFBP5	MDP	CD13	MDP	CD13	MDP
Cluster 43	CD13	CD13	CD13	SPR2	IGFBP5	MDP	CD13	MDP	CD13	MDP

Fig. 46. Top 10 significant markers for clusters after correction

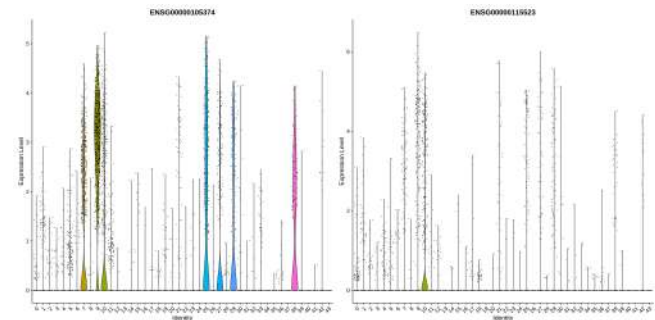
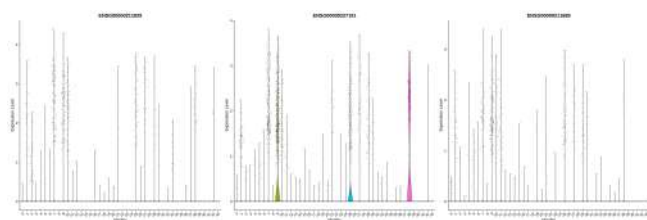


Fig. 47. NK cell markers in clusters before correction  
NKG7 (ENSG00000105374), GNLY (ENSG00000115523)

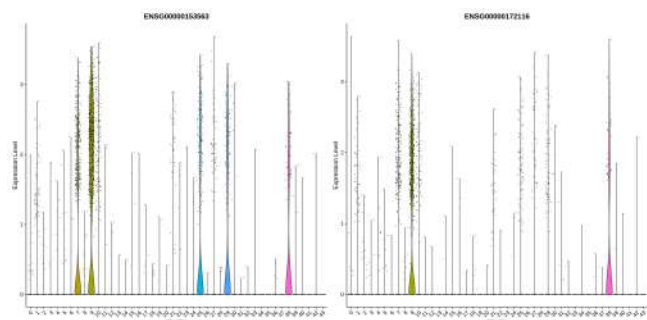
**Identity by cell type markers.** The NK cell markers: NKG7 (ENSG00000105374), GNLY (ENSG00000115523) were highly expressed in cluster 9, 10, 25 and 38 as seen on 47.

When analyzing  $\gamma\delta$  T cells markers: TRDC (ENSG00000211829), TRGC2 (ENSG00000227191) and TRGC1 (ENSG00000211689) we found that clusters 9, 25 and 38 had high expression levels of these markers (Figure 48).





**Fig. 48.**  $\gamma\delta$  T cell markers in clusters after correction  
TRDC (ENSG00000211829), TRGC2 (ENSG00000227191) and TRGC1 (ENSG00000211689)



**Fig. 49.** CD8+ T cell markers in clusters after correction  
CD8A (ENSG00000153563), CD8B (ENSG00000172116)

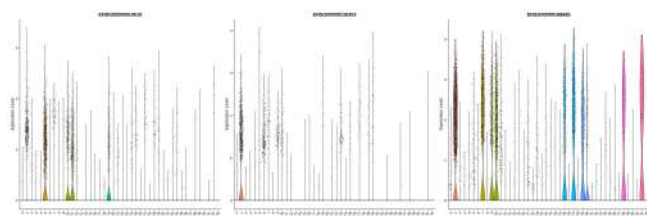
Another set of canonical markers we analyzed were CD8+ T cell markers: CD8A (ENSG00000153563), CD8B (ENSG00000172116). They were present in the highest levels in 9 and 38 clusters, which can be seen on [Figure 49](#).

In this case CD4+ T cells markers: CD4 (ENSG00000010610), CCR7 (ENSG00000126353), IL7R (ENSG00000168685) allowed us to identify naive CD4+ T (CCR7+) population as mainly cluster 1 and memory CD4+ T (CCR7-) population in clusters 5, 10, 11 and 19. ([Figure 50](#)).

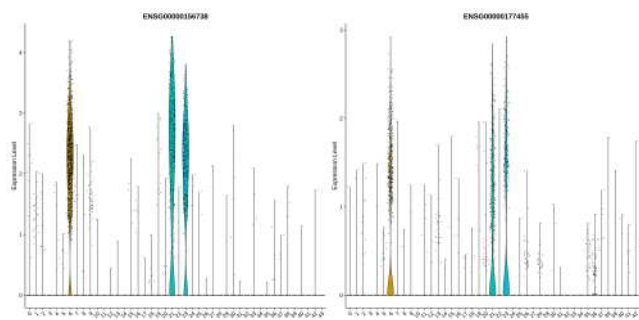
Canonical markers of B cells: MS4A1 (ENSG00000156738), CD19 (ENSG00000177455) were found in clusters 6, 21 and 23 as seen on [Figure 51](#).

After correction for sample number mast cell markers: TPSAB1 (ENSG00000172236), TPSB2 (ENSG00000197253), GATA1 (ENSG00000102145), GATA2 (ENSG00000179348) had highest expression levels in cluster 33 as seen on [Figure 52](#).

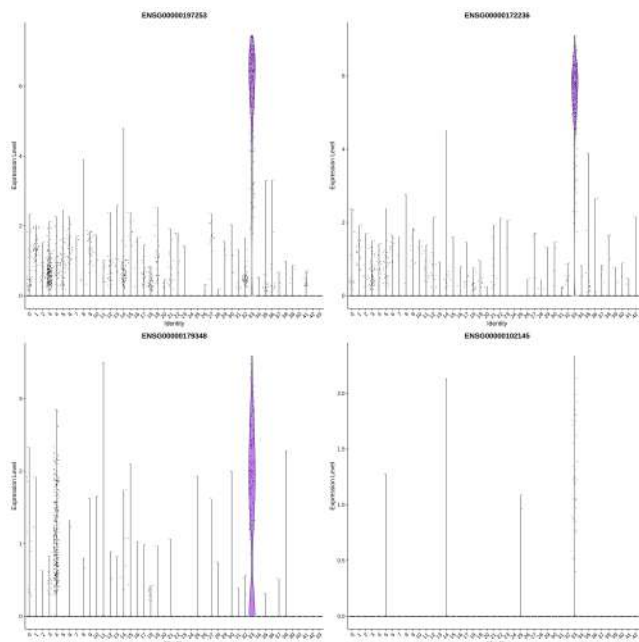
While checking in which clusters high levels of monocytes marker mRNA were present we found it hard to differentiate between CD14+ and CD16+ clusters. Markers used were then same as before CD14+ monocytes:



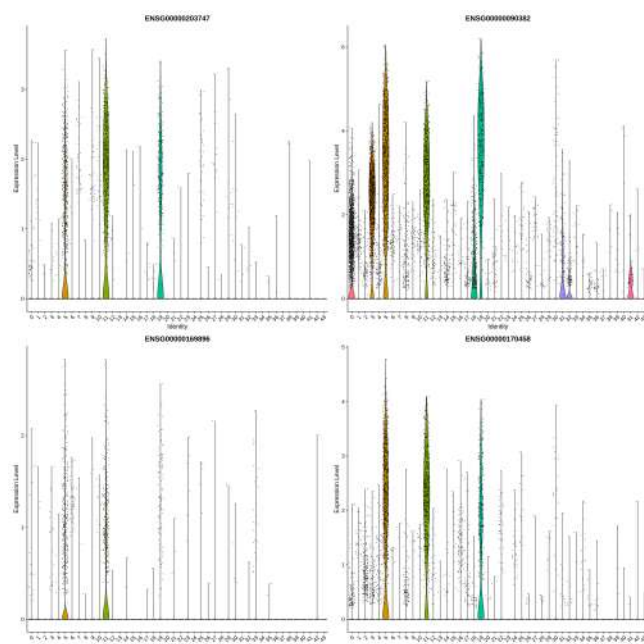
**Fig. 50.** CD4+ T cell markers in clusters after correction  
CD4 (ENSG00000010610), CCR7 (ENSG00000126353), IL7R (ENSG00000168685)



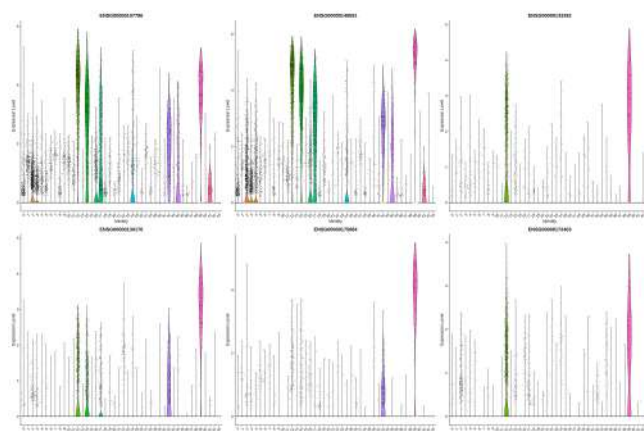
**Fig. 51.** B cell markers in clusters after correction  
MS4A1 (ENSG00000156738) and CD19 (ENSG00000177455)



**Fig. 52.** Mast cell markers in clusters after correction  
From top left to bottom right: TPSB2 (ENSG00000197253), TPSAB1 (ENSG00000172236), GATA2 (ENSG00000179348), GATA1 (ENSG00000102145)



**Fig. 53.** Monocytes markers in clusters after correction  
From top left to bottom right: FCGR3A (ENSG00000203747), LYZ (ENSG00000090382), ITGAM (ENSG00000169896), CD14 (ENSG00000170458)



**Fig. 54.** Smooth muscle cells markers in clusters before correction  
From top left to bottom right: ACTG2 (ENSG00000163017), TAGLN (ENSG00000149591), MYH11 (ENSG00000133392), CNN1 (ENSG00000130176), DES (ENSG00000175084), SYNPO2 (ENSG00000172403)

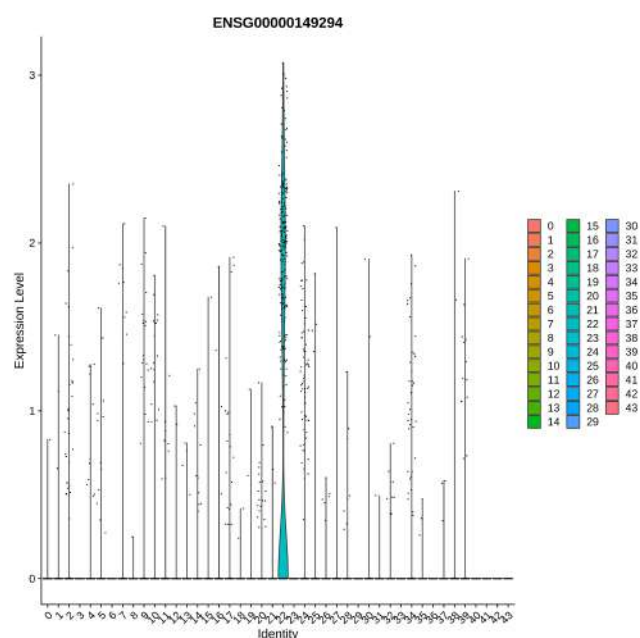
CD14 (ENSG00000170458), CD16+ monocytes: FCGR3A (ENSG00000203747) general monocytes markers: ITGAM (ENSG00000169896) and LYZ (ENSG00000090382).

The highest levels of marker mRNA were present in clusters 5, 11 and 19 as can be seen on [Figure 53](#).

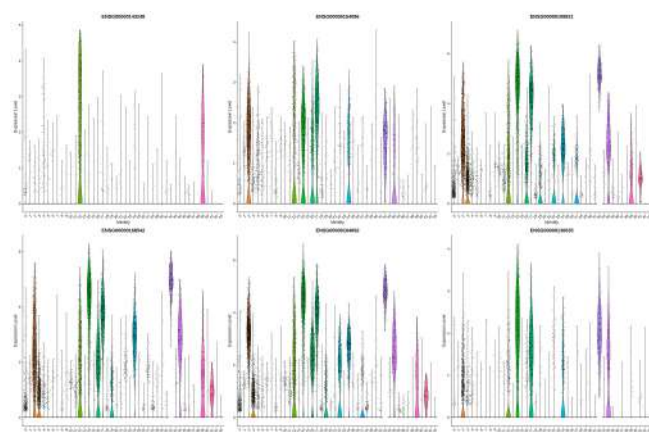
For comparison to data before correction we checked expression levels of smooth muscle markers: ACTG2 (ENSG00000163017), TAGLN (ENSG00000149591), MYH11 (ENSG00000133392), CNN1 (ENSG00000130176), DES (ENSG00000175084), SYNPO2 (ENSG00000172403) and found they were present most consistently in clusters 12, 32, 39 ([Figure 54](#)).

Neuronal cell marker NCAM1 was found highly expressed in cluster 22 [Figure 55](#).

Another cell type we checked for comparison with clustering



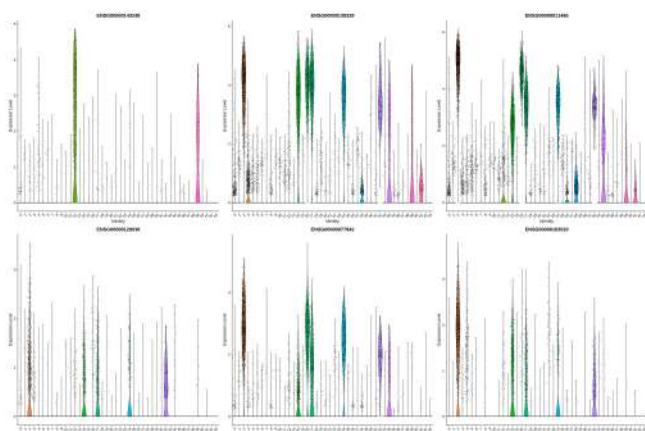
**Fig. 55.** Neuronal cell marker NCAM1 (ENSG00000149294) in clusters after correction



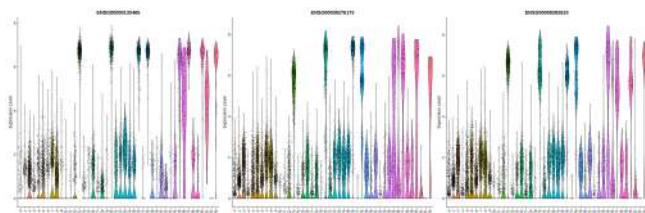
**Fig. 56.** Fibroblasts markers part 1 in clusters after correction  
From top left to bottom right: RGS5 (ENSG00000143248), THY1 (ENSG00000154096), COL1A1 (ENSG00000108821), COL3A1 (ENSG00000168542), COL1A2 (ENSG00000164692), COL5A1 (ENSG00000130635)

results before correction for sample were fibroblasts. Again we used positive markers: THY1 (ENSG00000154096), COL1A1 (ENSG00000108821), COL3A1 (ENSG00000168542), COL1A2 (ENSG00000164692), COL5A1 (ENSG00000130635) ([Figure 56](#)) and LUM (ENSG00000139329), DCN (ENSG0000011465), LOXL1 (ENSG00000129038), FBLN1 (ENSG00000077942), FBLN2 (ENSG00000163520) ([Figure 57](#)) and negative marker RGS5 (ENSG00000143248) which is not expressed in fibroblasts ([12](#)). Markers expression indicates that fibroblast population is most likely located in clusters 2, 14, 32 and 34.

**Identity by cluster markers.** Similarly as in clustering results before data correction we were able to identify IgA+ plasma cells by analyzing markers IGHA1



**Fig. 57.** Fibroblasts markers part 2 in clusters after correction  
From top left to bottom right: RGS5 (ENSG00000143248), LUM (ENSG00000139329), DCN (ENSG00000011465), LOXL1 (ENSG00000129038), FBLN1 (ENSG00000077942), FBLN2 (ENSG00000163520)



**Fig. 58.** IgA+ plasma cell markers in cluster 13 after correction JCHAIN (ENSG00000132465), IGHA2 (ENSG00000276173), IGHA1 (ENSG00000282633)

(ENSG00000282633), IGHA2 (ENSG00000276173) and JCHAIN (ENSG00000132465) as markers which were identifying cluster 13 (Figure 58).

**Comparison with ScType results.** In this case the classification diverged. However most classification were similar enough that the differences can be explained by choice of markers.

In both cases cluster 1 was assigned naive CD4+ T cell type. Clusters that were classified by us as B cells, ScType classified as pre-B cells and as naive B cells clusters 43, 40, 28, 13, 37 and 35. So our classification of cluster 13 as IgA+ plasma cells was most likely correct.

As non-classical monocytes ScType marked only cluster 19, while cluster 11 was identified as macrophages and cluster 5 as dendritic cells.

Further comparison can be seen on Figure 59 and the distribution of clusters between tissues is shown on 60.

## Discussion

Single-cell 'RNA sequencing analysis is a complex subject that without depending on prior knowledge about the cells and their expression patterns as well as markers that might help to identify them. For that reason most research tries to merge information from different types of sequencing for example bulk RNA sequencing and sc-RNA-seq. In cancer research this approach allows to classify cells by their CMS type. However, if one studies only sc-RNA-seq data, classification of unsupervised clusters is a multifaceted problem that requires choosing appropriate markers to differenti-

ate groups based on current knowledge about transcriptomics map of cancer tissues. This clearly underlines the need of such databases as Protein Atlas.

Generally identification of cell populations relies on manual annotation of cell clusters using established transcriptomic markers. It is unquestionably a time consuming process and relies on proper selection of markers for specific dataset. Finding informative markers for both individual cell clusters as well as various cell types present in the sample.

Using platforms such as ScType that enable fully automated cell type classification is definitely a more effective approach, especially if we are interested in general classification that would later allow us to explore cell subtypes further.

Another important thing that can be noted based on our results is the fact that as we previously stated, trying to correct data for possible batch effects/variation from non biological variables is not always beneficial for the sake of interpretability of our results. After regressing our data for sample number we obtained a larger number of seemingly better separated clusters, however it didn't help with the classification. We can even say that assigning cluster identity was easier before data correction.

All of the above observations only confirm that sc-RNA-seq data analysis and unsupervised clustering are not straightforward problems and require a lot of thought and background knowledge about the data and studied processes.

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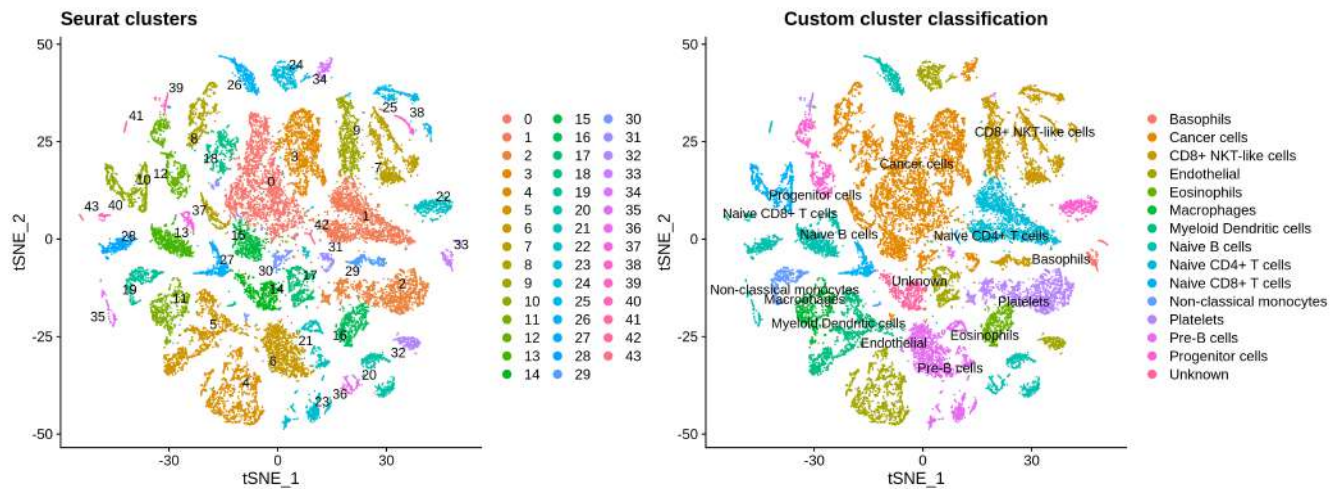


Fig. 59. Seurat clusters vs ScType cluster identities after correction

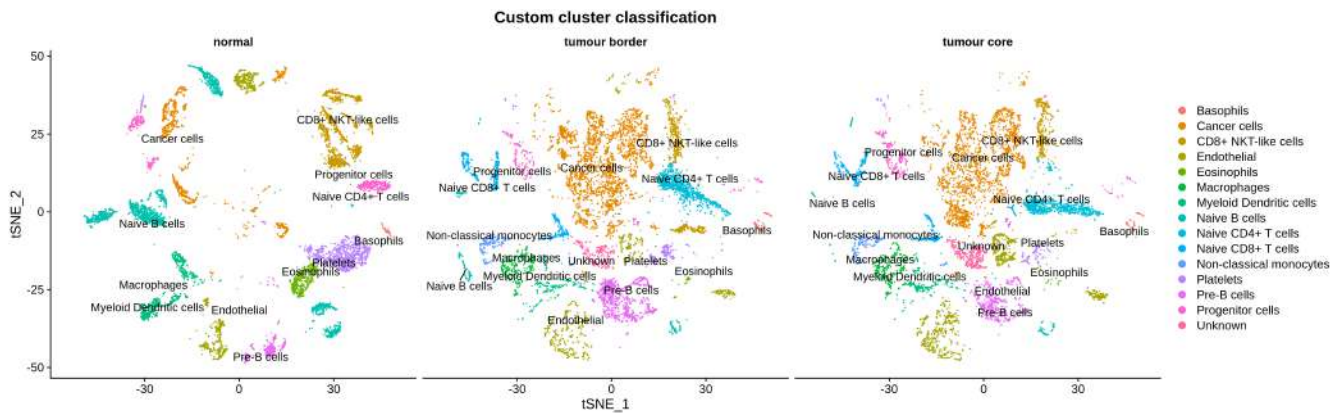


Fig. 60. ScType cluster identities by sampling site after correction

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