Rebecca Suomi

Single-cell RNA-sequencing in novel preventative and therapeutic applications in breast cancer- related lymphedema

Syventävien opintojen kirjallinen työ

Kevätlukukausi 2024

Rebecca Suomi

Single-cell RNA-sequencing in novel preventative and therapeutic applications in breast cancer related lymphedema

Kliininen laitos

Kevätlukukausi 2024

Ohjaaja: EL Susanna Pajula

TURUN YLIOPISTO
Kliininen laitos, Lääketieteellinen tiedekunta
SUOMI, REBECCA: Yksisolu-RNA-sekvensoinnin hyödyntäminen rintasyöpähoitojen jälkeisen imunesteturvotuksen ennaltaehkäisyssä ja hoidossa
Syventävien opintojen kirjallinen työ sekä suomenkielinen tiivistelmä, 53 sivua
Plastiikkakirurgia
Maaliskuu 2024

Turun yliopiston laatujärjestelmän mukaisesti tämän julkaisun alkuperäisyys on tarkastettu Turnitin OriginalityCheck -järjestelmällä.

Tiivistelmä

Yksisolu-RNA-sekvensointi (scRNA-seq) kuuluu uuden sukupolven sekvensointimenetelmiin (new generation sequencing, NGS). Sillä voidaan kerätä ja analysoida suuria datamääriä yksittäisten solujen ribonukleiinihappo (RNA) -aktiivisuudesta huomattavasti aiempia menetelmiä tarkemmin. Menetelmällä voidaan tunnistaa harvinaisia ja yksityiskohtaisia muutoksia yksittäisten solujen vasteesta esimerkiksi erilaisissa sairaustiloissa.

Rintasyöpähoitojen yhteydessä tehtävät kainaloiden imusolmukepoistot sekä paikalliset säde- ja kemoterapiahoidot vaurioittavat imunestekiertoa sekä aiheuttavat usein imunesteturvotusta yläraajaan, eikä pitkäaikaiselle imunesteturvotukselle ole vielä saavutettu hyvää ennaltaehkäisevää tai parantavaa hoitovastetta. Hyödyntämällä yksisolu-RNA-sekvensointia imunesteturvotuksen tutkimisessa, voidaan löydöksiä mahdollisesti hyödyntää tulevaisuuden kohdennettujen hoitomuotojen kehittämisessä.

Kirjallisuuskatsauksessa on koottu scRNA-seq tutkimuksia sekundaarisen imunesteturvotuksen patofysiologiasta. Tuloksissa muun muassa CD4+-solulinja sekä *TREM1*-reseptori omaavat tärkeän roolin tulehdusprosessin etenemisessä ja rasva- ja sidekudosmuodostuksessa. *LPL*-geeni ja PPAR-gamman lisääntynyttä ilmentymää on havaittu rasvakudoksen hypertrofian taustalla ja *CLEC3B*-geenin ilmentymää fibroosissa. VEGF ja PDGFD-viestintä olivat keskeisiä tulehdus- sekä rasva- ja sidekudosmuutosten osalta.

Tulevaisuudessa olisi tärkeää selvittää, ilmeneekö keskeisiä merkkiaineita tai muita määritettäviä solu-, molekyyli- tai RNA-tason muutoksia jo ennen kliinisesti havaittavan imunesteturvotuksen kehittymistä. Kirurgiset ja lääkkeettömät hoitomuodot tulevat säilyttämään roolinsa, mutta yksisolu-RNA-sekvensoinnin avulla voidaan kohdentaa uusia hoitomuotoja lääkekehityksen osalta.

UNIVERSITY OF TURKU
Faculty of medicine
SUOMI, REBECCA: Single-cell RNA-sequencing in novel preventative and therapeutic applications in breast cancer related lymphedema
Literary work of advanced studies, 53 pages
Plastic surgery
March 2024

The originality of this thesis has been checked in accordance with the University of Turku quality assurance system using the Turnitin OriginalityCheck service.

Abstract

Single-cell RNA-sequencing (scRNA-seq) is a form of new generation sequencing (NGS). It can be used to gather and analyse large datasets of single cells involving ribonucleic acid (RNA) -activity with precision and efficiency unattained by previous methods. scRNA-seq can be used to ascertain rare and detailed changes in a cell's transcriptomic and proteomic activity in varying environments. In a clinical setting, it can be used to identify or guide new potential targeted therapies.

Axillary lymph node dissection and biopsy, as well as radiative treatments and chemotherapy in conjunction with breast cancer surgery damage the axillary lymphatic system and heighten the risk of breast cancer-related lymphedema (BCRL) in the upper extremity. No curative or preventative treatment exists to date. Utilization of scRNA-seq could lead to new solutions regarding pharmacotherapeutic development in chronic secondary lymphedema.

The aim of this literature review was to compile previous cellular and molecular information regarding lymphedema and present results gained about secondary lymphedema pathophysiology using scRNA-seq methods. scRNA-seq results suggest a pivotal role of CD4+-cells and *TREM1*-receptor overexpression in chronic inflammation, adipose hypertrophy, and fibrosis. A heightened expression of the *LPL*-gene and PPAR-gamma lead to adipose hypertrophy and the *CLEC3B*-gene in fibrotic progression. Dysregulated VEGF and PGFD signaling was central in inflammation, adipose hypertrophy, and fibrosis.

Future studies would benefit from determining, whether molecular or cellular changes occur prior to discernible lymphedema. Surgical and conservative treatments have steadily secured a role in secondary lymphedema treatment, but scRNA-seq holds vast potential in developing novel targeted pharmacotherapeutic solutions for BCRL.

Table of Contents

LIST OF ABBREVIATIONS 3

2. INTRODUCTION 5

3. APPLICATIONS AND DEVELOPMENT OF SCRNA-SEQ IN CELL ANALYSIS AND PATHOPHYSIOLOGY 7

3.1 OVERVIEW OF SCRNA-SEQ 7

3.2 THE USE OF SCRNA-SEQ IN CLINICAL STUDIES 8

3.3 PROCESSING AND AMPLIFICATION OF SINGLE-CELL OR SINGLE-NUCLEI RNA-DATA 8

3.3.1 Barcoding cells and transcripts to ease downstream analysis *9*

3.3.2 Amplification of complementary DNA and further processing steps *10*

3.4 UPSCALING SCRNA-SEQ TO A COMMERCIALLY VIABLE LEVEL 11

3.4.1 Split Pool Ligation-based Transcriptome sequencing *12*

3.4.2 Droplet-based systems *13*

3.5 CONTAINING ERROR MARGINS AND QUALITY CONTROL OF SCRNA-SEQ DATA 13

3.5.1 Quality control and guidelines for assessment of data viability *14*

3.5.2 Batch effects in scRNA-seq *16*

3.6 POST-PROCESSING STEPS OF SCRNA-SEQ DATA 17

3.6.1 Principal component analysis *17*

3.6.2 Clustering *18*

3.6.3 Mapping cell-cell communications mediated by ligand-receptor complexes *19*

4. BREAST CANCER-RELATED LYMPHEDEMA, POST-TREATMENT SECONDARY LYMPHEDEMA DEVELOPMENT ON A MACROSCOPIC LEVEL AND OVERVIEW OF CURRENT TREATMENT METHODS **20**

4.1 THE LYMPHATIC SYSTEM 20

4.1.1 Development of lymphedema and symptoms *21*

4.2 HISTOLOGICAL CHANGES IN LYMPHATIC VESSELS FOLLOWING LYMPHEDEMA 22

4.3 LYMPHEDEMA STAGING 23

4.4 BREAST CANCER-RELATED LYMPHEDEMA FOLLOWING DAMAGE TO LYMPHATIC TISSUE 24

4.4.1 Secondary complications of chronic lymphedema *24*

4.5 CELLULAR AND MOLECULAR CHANGES IN THE STROMAL VASCULAR FRACTION IN LYMPHEDEMA USING TRADITIONAL CELL ANALYSIS METHODS 25

4.6 CURRENT TREATMENT METHODS OF SECONDARY LYMPHEDEMA 27

4.6.1 Pharmacotherapeutic approaches in lymphedema *28*

4.6.2 Combining microsurgery with growth factor therapy *29*

5. LYMPHEDEMA PATHOPHYSIOLOGY DESCRIBED AT A CELLULAR AND MOLECULAR LEVEL USING SCRNA-SEQ **30**

5.1 BULK RNA-SEQUENCING AND LYMPHEDEMA PATHOPHYSIOLOGY 31

5.2 USING SCRNA-SEQ TO MAP OUT PATHOPHYSIOLOGIC CHANGES AND CELL INTERACTIONS IN LYMPHEDEMA 31

5.2.1 Single cell transcriptional profiling of adipose derived stromal cells in upper extremity lymphedema 32

5.2.2 Single cell RNA sequencing of adipose tissue in lower extremity secondary lymphedema 33

6. DISCUSSION: INFORMATION GAINED USING SCRNA-SEQ IN CHRONIC SECONDARY LYMPHEDEMA IN COMPARISON TO TRADITIONAL METHODS **35**

6.1 CELL POPULATIONS INVOLVED IN LYMPHEDEMA 35

6.2 MOLECULAR CHANGES IN ADIPOSE TISSUE HYPERTROPHY IN LYMPHEDEMA 36

6.3 INVOLVEMENT AND POPULATION SIZE OF ASCS 36

6.4 FIBROTISATION AND DYSREGULATED SIGNALING AND DIFFERENTIATION PATHWAYS 37

6.5 VEGF AND PDGF IN LYMPHEDEMA 38

6.6 CONCLUDING REMARKS AND SYNOPSIS 38

7. MATERIALS AND METHODS **39**

8. STRENGTHS AND LIMITATIONS **39**

9. CONCLUSIONS **41**

10. BIBLIOGRAPHY ..... ERROR! BOOKMARK NOT DEFINED.

List of Abbreviations

ABBREVIATION	DEFINITION
ALND	Axillary lymph node dissection
ANG-1	Angiopoietin-1
ANG-2	Angiopoietin-2
ASC	Adipose-derived stromal cell
BCRL	Breast cancer-related lymphedema
CCAAT/EBP-ALPHA	Cytosine-cytosine-adenosine-adenosine-thymidine enhancer-binding-protein-alpha
CDNA	Complementary DNA
DC	Dendritic cell
DEG	Differentially expressed gene
DNA	Deoxyribonucleic acid
FACS	Fluorescence activated cell sorting
FDA	Food and Drug Administration
GSEA	Gene set enrichment analysis
HIF-1	Hypoxia inducible factor 1
HTS	High throughput sequencing
HVG	Highly variable genes
IL-1BETA	Interleukin 1-beta
ISL	International Society of Lymphology
IVT	In vitro transcription
LCM	Laser capture microdissection
LVA	Lymphaticovenous anastomoses
MACS	Magnetic activated fluorescence sorting
MNN	Mutual nearest neighbor
MRNA	Messenger RNA
NGS	Next-generation sequencing techniques
NK	Natural killer
NKT	Natural killer T
PCA	Principal component analysis
PCR	Polymerase chain reaction

PDGFD	Platelet derived growth factor D
PPAR-GAMMA	Proliferator-activated receptor-gamma
QC	Quality control
QOL	Quality of life
RNA-SEQ	RNA sequencing
RNA	Ribonucleic acid
SCRNA-SEQ	Single-cell RNA sequencing
SLNB	Sentinel lymph node biopsy
SNRNA-SEQ	Single nuclei RNA sequencing
SPLIT-SEQ	Split Pool Ligation-based Transcriptome sequencing
SVF	Stromal vascular fraction
T-SNE	t-stochastic neighbour embedding
TGF-BETA1	Tumour growth factor-beta1
TH1	T-helper 1
TH2	T-helper 2
TNF-ALPHA	Tumour necrosis factor-alpha
TREG	Regulatory T
UMI	Unique molecular identifier
UML	Unsupervised machine learning
VEGF-C	Vascular endothelial growth factor-C
VEGF-D	Vascular endothelial growth factor-D
VEGF	Vascular endothelial growth factor
VLNT	Vascularized lymph node transfer

2. Introduction

Ribonucleic acid sequencing (RNA-seq) is a genomic method of detecting and quantifying messenger RNA (mRNA) in a biological sample. It belongs to a series of next-generation sequencing (NGS) techniques, which revolutionized the transcriptomics field by simplifying workflow and allowing for more comprehensive transcriptome profiling than previous techniques.[1] While bulk RNA-seq has made studying purified cell populations of a single origin easier, they have lacked precision to analyze single cell expression and transitional differentiation as a continuum – instead averaging results, compromising heterogeneity and potentially masking rare cell populations and consequent transcriptomes.[2]

Single-cell RNA sequencing (scRNA-seq) was developed as a method to describe the activity of a single cell's transcriptional changes which vary depending on internal and external factors[3]. scRNA-seq can also be used in detecting rare cell populations or genetic expression, which otherwise would go unnoticed in a larger pool of cells, as is the case in bulk RNA-seq (see Figure 1).[4] Additionally, scRNA-seq can give information on the lineage and developmental stages of related and similar, but heterogenous cell types. Such states occur, for example, in cellular differentiation and development. [5]

scRNA-seq can be used in clinical settings to gain a better understanding of underlying pathophysiology. It has raised interest in the case of breast cancer-related lymphedema (BCRL), which is a prevalent issue affecting up to 42% of patients[6] following breast cancer related surgical, radio- and chemotherapeutic treatments.[7] Chronic BCRL causes fibrotisation[8,9] and adipose tissue hypertrophy[9] in the affected limb. Sensory, physiological, and mechanical changes in the limb lead to a significant decrease in quality of life (QoL).[10] However, the exact pathophysiology of BCRL is not well understood[11] and therefore precise treatment options have been difficult to develop. To date, no curative treatments[12] or Food and Drug Administration (FDA)-approved pharmacotherapeutic interventions exist.[13] scRNA-seq could potentially be used to both discern transcriptional changes in a secondary lymphedema environment and develop novel preventative or curative therapeutic methods based on targeted care.

The following text will provide an overview on scRNA-seq technology in its present state, methodology of scRNA-seq, as well as a description of data processing and analysis. We will be discussing the uses and implications of scRNA-seq in fibrosis and adipose tissue hypertrophy caused by chronic secondary lymphedema and discussing the relationship of these findings in BCRL and potential therapeutic approaches.

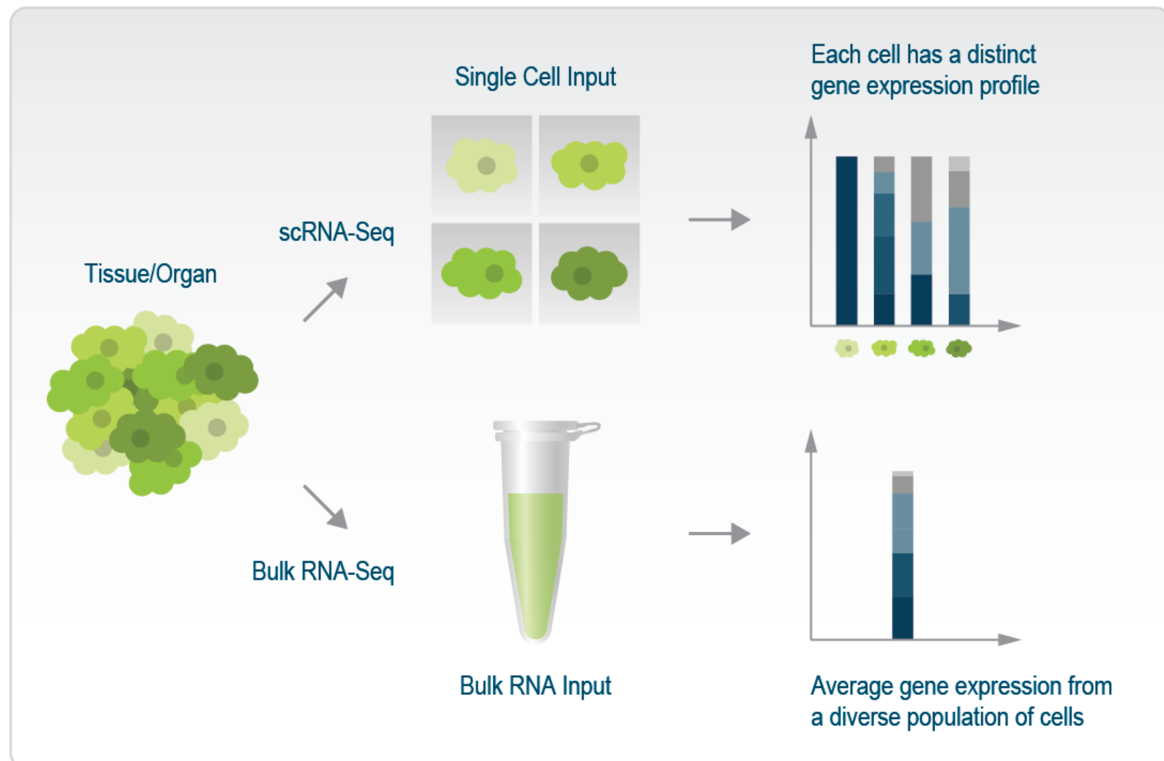

Figure 1. Visual representation of gene expression data from scRNA-seq and bulk RNA data *(Lexogen, 2024)*

3. Applications and development of scRNA-seq in cell analysis and pathophysiology

3.1 Overview of scRNA-seq

scRNA-seq was pioneered in 2009 by Tang et al.,[3] when the first scRNA-seq study was published. The study described the sequencing of a single mouse blastomere transcriptome with higher genetic yields, and significantly increased specificity than with previous microarray techniques. The technique has since been developed and has yielded promising results in the study of transcriptomics. Although scRNA-seq still remains a niche field of study, commercial availability of scRNA-seq techniques is now readily available and its potential is steadily being expanded upon, with both a significant decrease in cost and an exponential rise in the number of cells and transcriptomes sequenced in a single round of scRNA-seq[5,14] (see Figure 2).

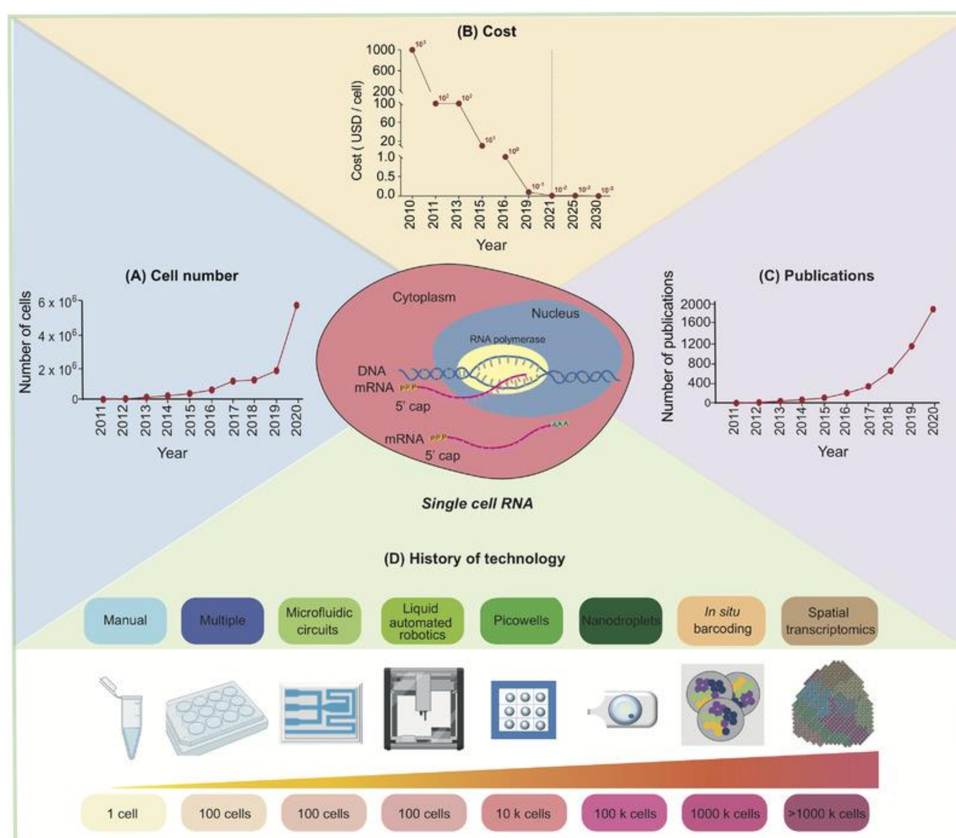

Figure 2. Developments in scRNA-seq from 2010-2020 *(Jovic et al., 2022)*

3.2 The use of scRNA-seq in clinical studies

scRNA-seq is valuable in clinical studies, as the attained data can be used to describe cell interactions, pathways, and up- or downregulation of cell activity.[5]The tumor microenvironment is especially an area of continual interest in scRNA-seq studies. New cell subtypes and subsets have been successfully identified in lung cancer,[15] detailed stromal and immune cell compositions have been recorded in breast cancer.[16] In pancreatic cancer, different immune cell compositions have been linked with differing survival rates and these interactions have been described with high immunologic detail, revealing novel heterogeneity. [17] scRNA-seq can also be used in describing the genetic details of tumor microenvironments with higher resolution than current genetic panels in head and neck cancers.[18] Rare, treatment resistant cell types can also be identified using scRNA-seq.[19]

3.3 Processing and amplification of single-cell or single-nuclei RNA-data

scRNA-seq begins with the identification and isolation of the cell of interest. Single cell isolation techniques (see Figure 3) include manual cell picking, fluorescence activated cell sorting (FACS), magnetic activated cell sorting (MACS), laser capture microdissection (LCM) and microfluidic approaches.[20]

Initially mRNA is identified from other RNA -such as ribosomal RNA- by its distinct poly(A) tail at the 3’ end of RNA by using a poly(T)-primer. After this step the poly(T)-primed mRNA is barcoded before converting it to complementary DNA (cDNA) and amplifying it to form a cDNA library.[4]

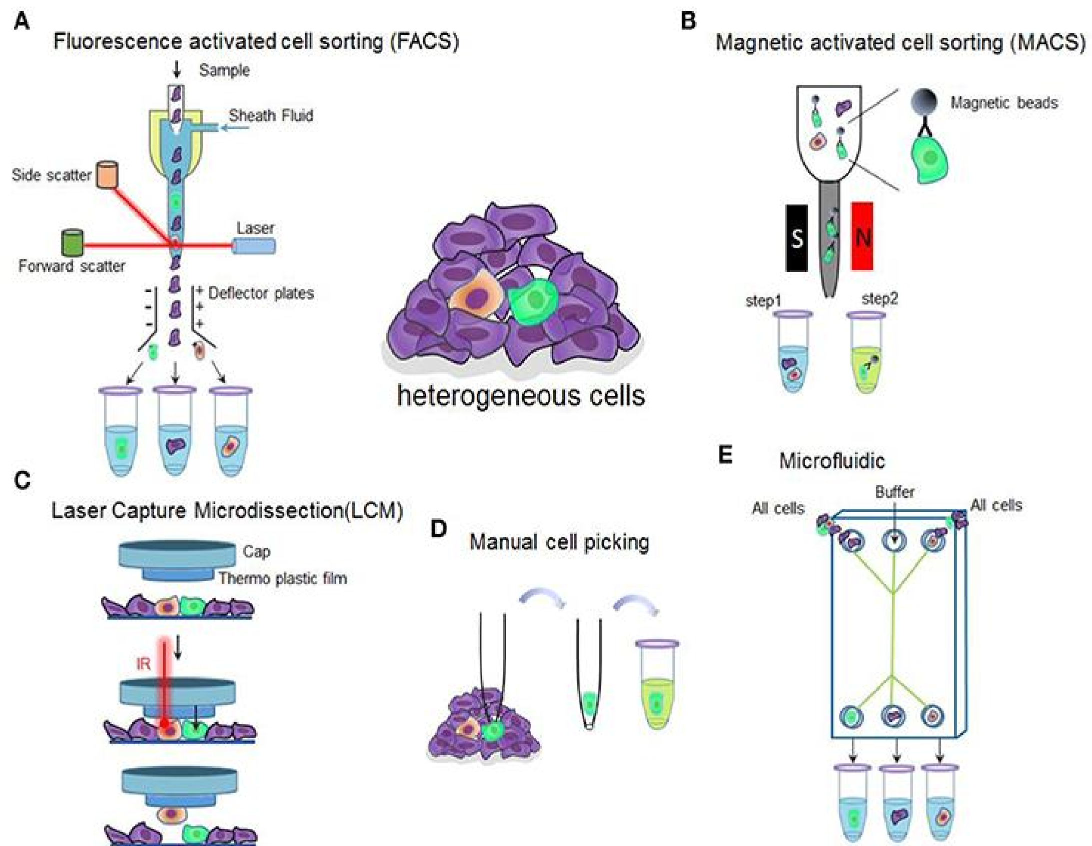

Figure 3. Single-cell isolation techniques described visually *(Hu et al., 2016)*

3.3.1 Barcoding cells and transcripts to ease downstream analysis

Cells of interest and transcripts can be barcoded by adding oligonucleotides called unique molecular identifiers (UMIs), usually approximately 5 – 16 bases in length[21,22], which serve as identification of the cell type in downstream analysis. It has been recommended, that 8 bases is the minimum length for UMIs as shorter sequences decrease robustness.[23] UMIs serve as a tool in post-processing data-analysis to identify and normalize amplification disproportionality and random variability that amplification inevitably causes. In high throughput sequencing (HTS), this is especially important when aiming to reduce quantification errors. UMIs are recommended to be used in low-input samples, deep sequencing (>80 million reads/sample) and detection of ultra-low frequency mutations.[24]

In droplet-based systems, cell-free RNA can constitute up to 20% of the eventual transcriptome and therefore cause significant bias, since these are not the transcripts which scRNA-seq aims

to study. Cell-free RNA causes contamination and can lead to incorrect and confounding conclusions. Cross-species spike-in cells can be used during the preparation workflow to counter this issue, since cross-species transcripts can be easily identified in later analysis. Spike-ins assume an even redistribution of transcripts in the sample. By standardizing and scaling data according to the fraction of measured contamination, data can be corrected to decrease bias.[25]

By combining both methods, molecular RNA spike-ins containing UMI-sequences can also be used to correct amplification bias. This technique has been suggested to be a gold standard in scRNA-sequencing, as using only UMIs without RNA spike-ins can also result in overestimation of RNA-expression, as counting errors can range from 5–400% in comparison to the baseline. Spike-ins therefore provide a benchmark for quality control (QC) and are recommended to be widely used in scRNA-seq experiments.[23]

3.3.2 Amplification of complementary DNA and further processing steps

After barcoding or adding other identifiable nucleotide sequences, mRNA is then reverse transcribed using reverse transcriptase enzymes to form cDNA[5] and amplified using polymerase chain reaction (PCR) or in vitro transcription (IVT) methods.[24]

In vitro transcription (IVT) can be used instead of PCR to amplify RNA and create cDNA libraries, and several sequencing methods utilize this currently as their protocol. PCR can introduce bias due to its many rounds of amplification, resulting in nucleic acid loss and increasing noise. Newer modified versions of IVT only include three rounds of linear amplification, which reduces the issues PCR can create when dealing with RNA material at such a small scale.[26]

After sequencing the amplified data, single-cell expression profiles can be formed, and cell types can be identified using various methods after several pre- and post-processing steps (see Figure 4).[27] Information regarding transcriptional activity and gene expression can then be analyzed and processed further.[5]

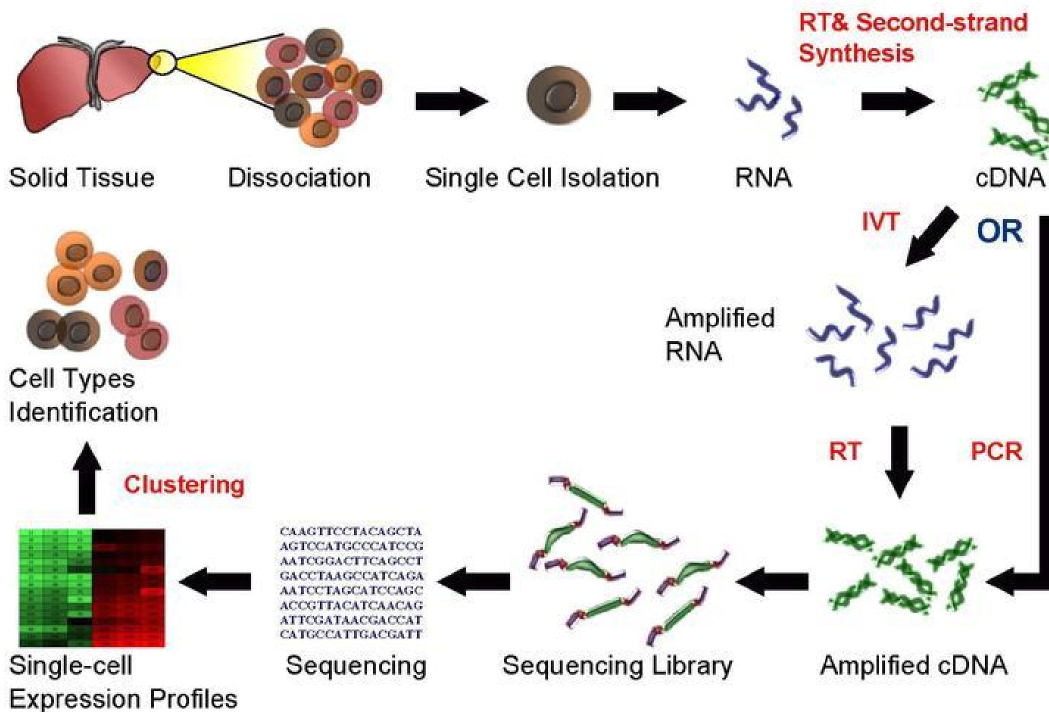

Figure 4. General overview of a simplified scRNA-seq workflow *(Yiyechern, 2014)*

3.4 Upscaling scRNA-seq to a commercially viable level

While the general workflow of scRNA-seq has remained the same, emerging techniques are now based on the combinatorial indexing or barcoding of single cells or nuclei, demonstrating a newer generation of scRNA-seq. The strength of these methods lies in their scalability, decreasing both cost and time, but also increasing throughput and increasing commercial availability.[28]

Therefore, single nuclei RNA sequencing (snRNA-seq)[29], 'split-pooling'[30] and droplet-based systems[31] have been developed, which allow for easier analysis of samples. For example, snRNA-seq aims to forgo mechanical stressors such as ultra-centrifugation and temperature elevation, and optimize the time needed by eliminating cell sorting and reducing manipulation time significantly.[32] snRNA-seq can be better suited for specific tissue or cell types, such as brain tissue, as single cells can be difficult to keep intact during dissociation.[14]

Advancements in these techniques have simplified and expanded processing possibilities, as well as decreased the cost significantly, making scRNA-seq commercially viable.[5] Figure 5 describes a variety of expanded scRNA-seq workflow systems which have been developed after the initial development of scRNA-seq in 2009. [2]

Method	Fluidigm C1 system (SMART-seq)	Fluidigm C1 system (mRNA Seq HT)	SMART-seq2	10X Genomics Chromium system	MARS-seq
cDNA coverage	Full-length	3′ counting	Full-length	5′/3′ counting	3′ counting
UMI	No	No	No	Yes	Yes
Amplification technology	Template switching-based PCR	Template switching-based PCR	Template switching-based PCR	Template switching-based PCR	*in vitro* transcription
Multiplexing of samples	No	Yes	No	Yes	Yes
Single cell isolation	Fluidigm C1 machine	Fluidigm C1 machine	FACS	10X Genomics Chromium single cell controller	FACS
Cell size limitations	Homogenous size of 5–10, 10–17, or 17–25 μM	Homogenous size of 5–10, 10–17, or 17–25 μM	Independent of cell size	Independent of cell size	Independent of cell size
Required cell numbers per run	≥10,000	≥10,000	No limitation	≥20,000	No limitation
Visual quality control check	Microscope examination	Microscope examination	No	No	No
Long term storage	No, must process immediately	No, must process immediately	Yes	No, must process immediately	Yes
Throughput	Limited by number of machines	Limited by number of machines	Limited by operator efficiency	Up to 8 samples per chip	Process is automated
Cost	+ + + + +	+ + +	+ + + +	+	+ +
Sample Preparation Scenario 1 (~5000 single cell)	Targeted cell No: 4992 cells	Targeted cell No: 4800 cells	Targeted cell No: 4992 cells	Targeted cell No: 5000 cells	Targeted cell No: 4992 cells
	26 rounds of 2 runs (2 C1 machines; concurrent)	3 rounds of 2 runs (2 C1 machines; concurrent)	26 rounds of 2 96-well plates	1 run	13 runs of 1 384-well plate
	~26 weeks	~3 weeks	~26 weeks	~2–3 days	~7 weeks
Sample Preparation Scenario 2 (~96 single cell)	Targeted cell No: 96 cells	Targeted cell No: Minimum 800 cell	Targeted cell No: 96 cells	Targeted cell No: Minimum 500 cells	Targeted cell No: 96 cells
	1 run (1 C1 machine)	1 run (1 C1 machine)	1 run of 96-well plates	1 run	1 run of 384-well plate
	~1 week	~1 week	~1 week	~2–3 days	~2–3 days

Figure 5. Single-cell RNA-sequencing methods *(See et al., 2018)*

3.4.1 Split Pool Ligation-based Transcriptome sequencing

One example of upscaling scRNA-seq is by using split pool ligation-based transcriptome sequencing (SPLiT-seq), which combines split pooling and combinatorial indexing. Benefits include increased efficiency, simplicity, and high yield. The method of split pooling forgoes other expensive and complex procedures, requiring only basic pipetting techniques and PCR for the indexing of transcriptomes. This type of sequencing facilitates the analysis of tens of

thousands of transcriptomes in a single process. Transcriptomes are further linked to their corresponding cell types by identifying specific genetic expression patterns.[30]

The original 96-well method -with three rounds of barcoding and the fourth round of barcoding combined with 24 rounds of PCR- can result in over 21 million unique barcode combinations, which can label over 1 million cells. Split pooling can be easily scaled, and specificity can be further improved by adding plates with more wells, or by introducing more rounds of barcoding.[30]

3.4.2 Droplet-based systems

Droplet-based systems are another example of upscaling scRNA-seq. They have been developed as an alternative to microfluidic and plate-based methods, enabling the analysis of tens of thousands of cells in a single application, with parallel sequencing also being possible.[31] Droplet-based methods such as the commonly used 10X Chromium system allow for bulk single-cell sequencing and reverse transcription takes place within each droplet. Gel beads containing primers, barcoded oligonucleotides, and specific adapters, combine with polyadenylated RNA.

An estimate of 50% of the cells that are loaded into the 10X Chromium system can be captured, therefore there is a partial loss in data occurring from this.[31] Transcriptome coverage is also more limited in droplet-based systems, as sequencing depth is compromised over the number of cells, and therefore less expressed genes are less likely to be captured. Also, only the 5' or 3' end of the transcript can be sequenced at a given time, but not both simultaneously.[33]

3.5 Containing error margins and quality control of scRNA-seq data

Once scRNA-seq raw data has been acquired, it must be processed through various means. This includes important steps such as quality control (QC), discarding data from poor-quality cells[34] and normalizing data.[35] Only after these steps is it favorable to proceed with the main analysis of attained data (see Figure 6) [36], as otherwise reliability of the results would be compromised.

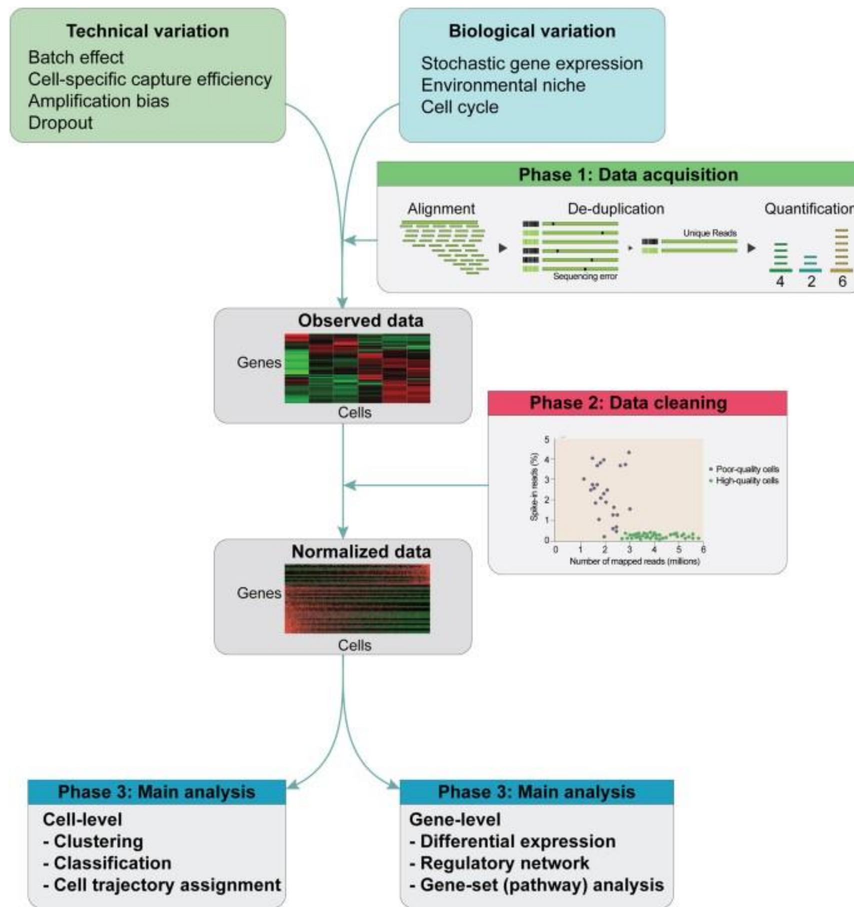

Figure 6. Schematic expression of scRNA-seq data pre-processing steps *(Hwang et al., 2018)*

3.5.1 Quality control and guidelines for assessment of data viability

Once data is obtained from scRNA-seq, it must be run through several QC metrics to determine which data is viable for use. Low quality cells are discarded from analysis. Metrics to consider are cell counts, UMIs per cell, genes per cell, UMI to gene counts ratio, mitochondrial counts ratio and novelty.[34]

Doublets can occur due to inaccurate capture or sorting of single cells during the initial stages of single cell processing. These happen more commonly in droplet-based systems, where cell counts are high. Doublets result in confounding data, where conclusions can be incorrectly made suggesting some intermediate or transitory cell-state, which may not actually exist. While

cut-off points are often determined regarding UMI or gene counts to normalize results and eliminate cells containing seemingly too many reads, automating the process can also erroneously lead to elimination of rare cells or transitory states. It has been suggested at this point in scRNA-seq development, that outliers should be inspected case-by-case.[34]

UMI or barcode counts can also be partially inaccurate in describing the number of transcripts or cells, as errors in barcode concentration and cell deaths can lead to higher counts than are truly present. In each experiment, it is important to be aware of the number of cells expected to be sequenced and be able to compare results to this. Additionally, it is important to know the expected cell capture efficiency of each method. Depending on the method, cell capture efficiency can range from 50-80%. UMIs corresponding to transcripts per cell should generally be >500. However, between 500–1000 UMIs per cell would suggest that a deeper sequencing would be preferable.[34]

Direct conclusions cannot be drawn simply by analysing the number of genes per cell. The reason behind this is because significant genetic variability can exist between cells, especially when considering pathophysiology applications of scRNA-seq. Also, cells can exist in transient and intermediate states as well as different sizes, and one aim of scRNA-seq is also to identify varying cellular states. However, a lack of gene counts can also indicate cell failure or destruction of cellular material. When attempting to identify singular cell types present in a population of sample cells, these are points to consider during analysis.[34]

It is advisable to compare UMI counts to gene counts per cell, as low-quality cells often contain low counts of both. However, high UMI counts paired with low-end gene counts can suggest the presence of a separate cell population of lower complexity, although it may also be an indication of dying cells. Mitochondrial counts yield further insight into whether the cells present are dying or dead. A high mitochondrial count ratio is often indicative of this. The ratio is high when gene and cell counts are low, generally leading to the conclusion that only mitochondrial mRNA is present in the sample, as cytoplasmic mRNA has leaked out of the cell at an earlier stage. In viable cells, the mRNA ratio is expected to be <0.2. However, the ratio threshold depends entirely on the type of cell observed. Cells involved in respiration will naturally contain higher counts of mitochondrial mRNA and are viable. Therefore, threshold values must be considered as indications, not absolutes.[34]

3.5.2 Batch effects in scRNA-seq

Batch effects are a well-known challenge in scRNA-seq and can raise issues, when aiming to determine actual biological similarities and differences between study samples.[37] Batch effects can be observed in clustering, if cells from different batches tend to cluster together, rather than clustering cells which are of the same cell type or origin.[37,38]

Batch effects are a result of variance from experimental designs and execution, such as different sequencing platforms, spatial and temporal effects in both methodology, laboratory environment and reagents.[39] Batch effects can be highly nonlinear and therefore can cause difficulties in standardizing different datasets.[40] This can cause major interpretation errors and therefore computational methods and algorithms, such as mutual nearest neighbor (MNN) matching, can be used to remove and minimize said effects (Figure 7).[37,41]

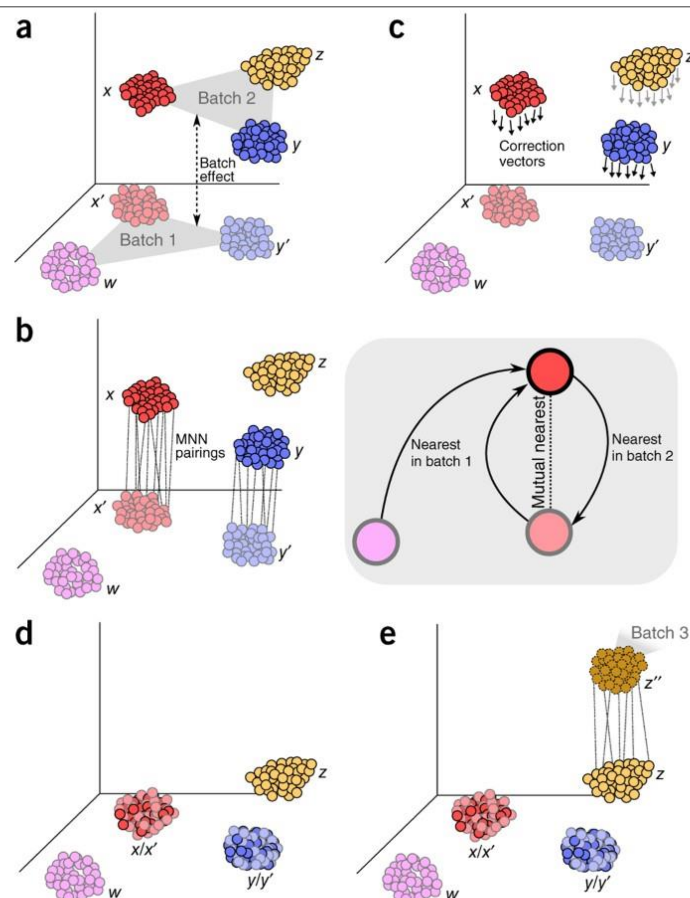

Figure 7. Batch effects and a visual representation of computational correction methods *(Haghverdi et al., 2018)*

3.6 Post-processing steps of scRNA-seq data

Transcriptomics yield large and complex raw datasets. This especially holds true when scaling up scRNA-seq by adding parallel runs and analysing tens of thousands of single-cell samples simultaneously. As scRNA-seq partially aims to identify cell responses of rare cell types or previously unidentified variations in transcription, data capture and analysis must retain enough detail to account for the identification of these potential -previously unknown- observations.

Therefore, data analysis software is in an important position in both analysing and visualizing the obtained data. Results can be altered significantly by the choices made in reducing dimensionality[42] and normalization[35] prior to running data through processing software. scRNA-seq protocols generally follow pipelines, which involve several steps of post-processing before actual data analysis can take place.[34]

3.6.1 Principal component analysis

Principal component analysis (PCA) algorithms are used in scRNA-seq for various purposes but are especially popular in dimensionality reduction before clustering[42]. PCA can be used to describe relationships between data points in multivariate datasets, and therefore is popular in scRNA-seq. PCA is used in quality control[43], visualization[44], rare cell type detection, cell similarities and cell type identification[45], batch effect removal[46] and many more. PC-scores are furthermore utilized in clustering and non-linear dimensionality reduction.[47] However, because of the vast amount of data points in scRNA-seq, PCA can be time consuming and require robust computing power. Even then, data analysis must be simplified, which leads to a loss of accuracy and potentially loss of differential gene expression in the process. PCA algorithms can differ significantly, and the choice of algorithm can lead to significantly inaccurate results.[47]

Unsupervised machine learning (UML) methods -which include dimensionality reduction and clustering- aim to connect attained data to correctly identified cell types. PCA is also widely used as an UML algorithm in scRNA-seq data processing.[47]

3.6.2 Clustering

Clustering has been widely used as an analysis and visualization method in scRNA-seq. Clustering results in a representation of subsets within the cells, as genetic similarities group together. Subsets from another cell are dissimilar, and therefore cluster separately (Figure 8).[48] Its strengths lie in the visualization of similarities and differences, approachability, and simplification of datasets.[33]

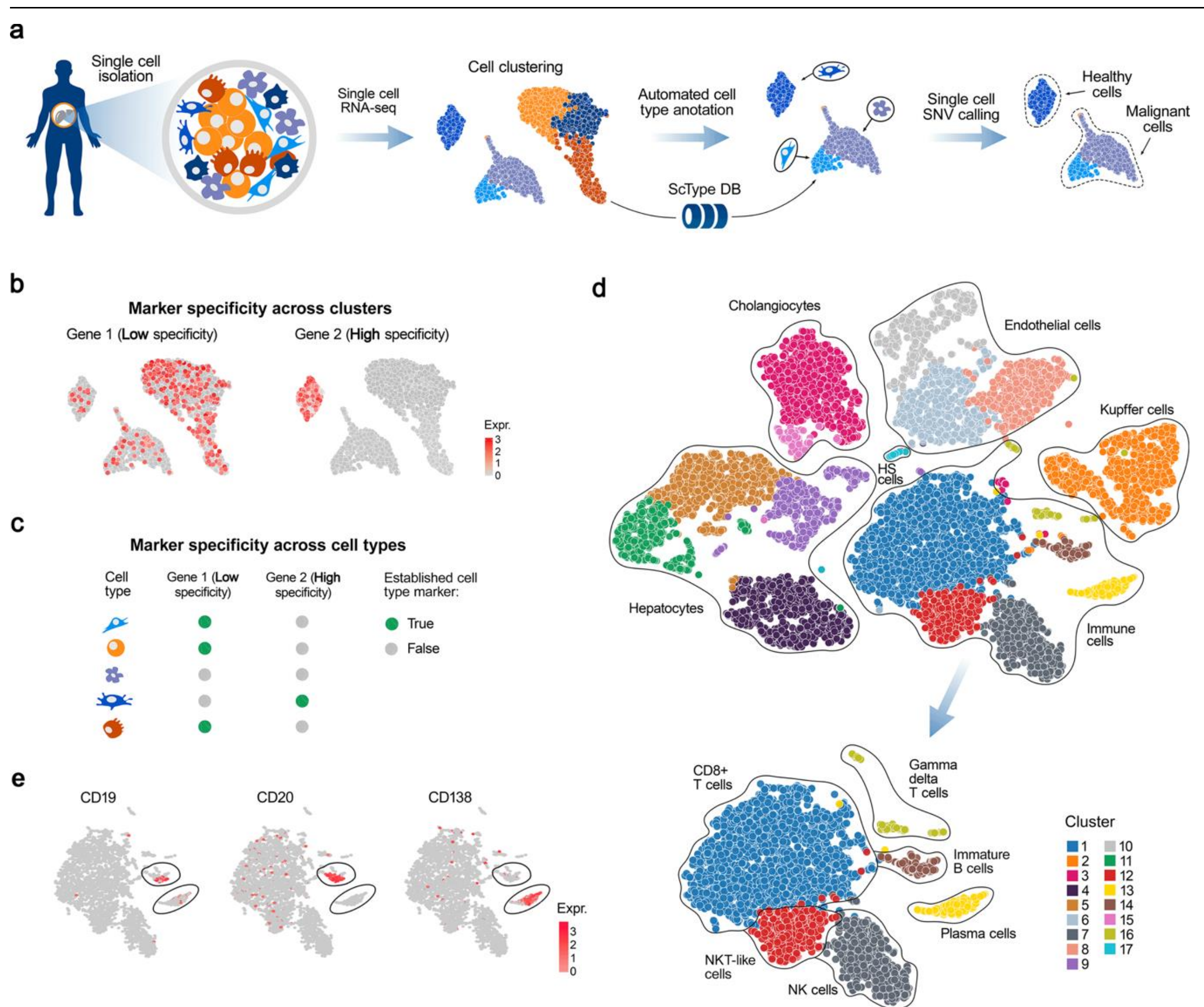

Figure 8. A comprehensive description of clustering and various steps of cell and cell marker identification in scRNA-seq *(Ianevski et al., 2022)*

Clustering, however, does not describe transitional states well. These states can be unintentionally disregarded owing to the nature of clustering analysis, as can be seen in Fig. 8d.[48] Also, clustering itself does not provide additional information about the quality of cells and how subsets relate to each other. Alternate analysis based on identifying molecular, genetic, or other cell markers must be undertaken to confirm cell types within clusters. Pre-processing steps are also critical when considering the accuracy of clustering results.[33]

3.6.3 Mapping cell-cell communications mediated by ligand-receptor complexes

When investigating connections between different cell types in a single dataset, scRNA-seq benefits from the analysis of cell-cell communication analysis. Analysis software such as CellPhoneDB v.2.0[49] combine previous knowledge of ligand and receptor subunit architecture with a framework predicting cell-cell communications from transcriptome data.

4. Breast cancer-related lymphedema, post-treatment secondary lymphedema development on a macroscopic level and overview of current treatment methods

4.1 The lymphatic system

The lymphatic system works in conjunction with the cardiovascular system to maintain a balance between removal and supply of fluid, removing metabolic waste and supplying nutrients to cells within the body (see Figure 9).[50,51]

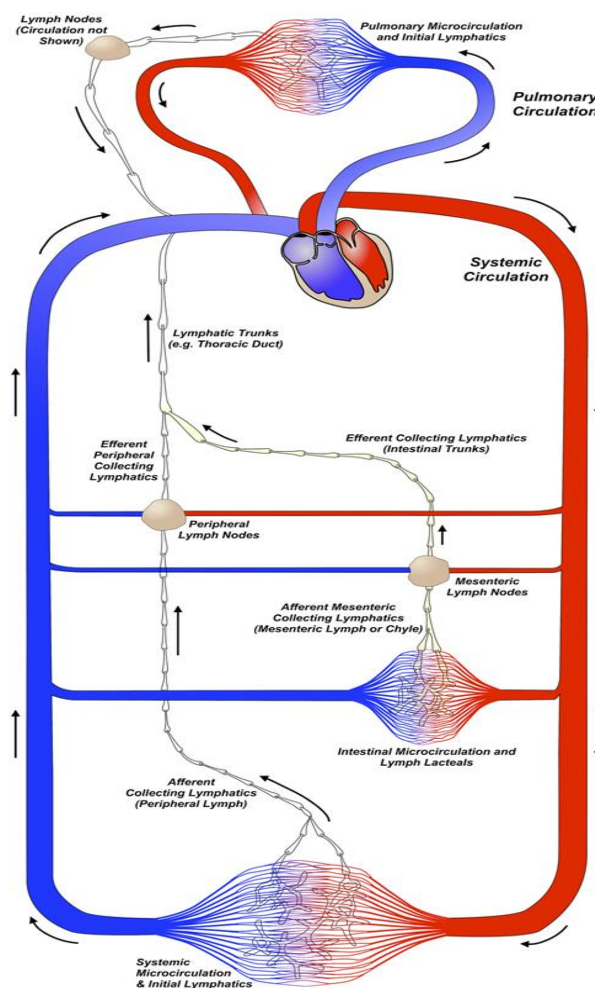

Figure 9. A visualization of the relationship between the lymphatic and vascular system *(Breslin et al., 2018)*

As blood circulates within arteries and veins, approximately 10% of circulating fluid transfers to the intracellular space and forms the lymphatic fluid.[52] This fluid contains debris and structures such as bacteria, viruses, metabolic waste, proteins, and fat.[52] Lymphatic fluid travels through lymphatic capillaries to larger vessels, which then filter through lymph nodes while circulating towards the heart and draining into large lymphatic ducts and finally back into the bloodstream.[51] While filtering lymphatic fluid, lymph nodes also capture possible cancerous cells and therefore can serve as platforms for cancerous cell proliferation.[50] Lymphatic fluid from the chest and upper extremities passes through respective axillary lymph nodes.[50]

4.1.1 Development of lymphedema and symptoms

Lymphedema is caused by lymphatic transport dysfunction. Interstitial fluid is a result of arterial capillary efflux. Interstitial fluid which is not absorbed by venous capillaries is absorbed as lymph into lymphatic vessels, finally transported back to the blood stream. Disruptions to the lymphatic system results in stasis, as the vessel capacity is overrun. Protein accumulation in the interstitium also increases colloid osmotic pressure and further results in fluid accumulation.[53] Lymphedema can be classified as either primary or secondary, where primary lymphedema originates idiopathically due to erroneous lymphatic development, and secondary lymphedema is a result of acquired injury to an otherwise normally developed lymphatic system.[54]

Lymphedema typically has a gradual onset and progresses with time. Early symptoms may include sensory changes such as tingling or numbness, or a slight increase in extremity circumference. Symptoms may initially vary.[50] Lymphedema can cause pain, changes in skin texture and discoloration, and limit mobility or decrease flexibility or the affected area.[55] The resulting symptoms have been shown to significantly decrease quality of life (QoL) both mentally and physically and potentially result in lifelong treatments as well as further invasive procedures to reduce swelling.[10]

4.2 Histological changes in lymphatic vessels following lymphedema

Mihara et al.[56] (2012) studied the progression of histological changes in collecting lymphatic vessels following gynecologic cancer intervention with lymphadenectomy with and without radiotherapy, resulting in lower extremity lymphedema. The progression of lymph vessel dysfunction was classified as four types; 1) normal, 2) ectasis, 3) contraction and 4) sclerosis. As lymphedema progressed, microvascular networks accompanying lymphatic vessels diminished. In early stages, the lymph vessel lumen was dilated due to an increase in endolymphatic pressure and the smooth muscle layer and collagen fibers thinned and elongated. Desmosomes gradually weakened between endothelial cells, exposing fibrous components to the lumen of the vessel.

Smooth contractile muscle cells transformed into synthetic muscle cells, migrated beneath the vessel endothelium, and caused a thickening of the wall. The synthetic smooth muscle produced growth factors and enzymes involved with a remodeling of the extracellular matrix. Collagen fibers were thickened, causing a significant narrowing of the lumen. Lymphatic vessels gradually progressed through the four types, finally losing elasticity and peristaltic movement to facilitate lymph transportation. In the sclerotic type, the lumen became almost or completely occluded, with lymphatic leakage into the extracellular tissue. Cellulitis and lymphangitis developed in chronic cases, leading to sclerotization. The study underlined the importance of early intervention in lymphedema.[56]

It is clear, that late stages of lymphedema cannot be addressed by pharmacotherapeutic agents or conservative treatment alone, as there already then exists irreparable damage to lymphatic function. However, it has been postulated, that lymphatic stasis alone cannot be the sole source behind the tissue injury associated with lymphedema, but inflammation instead may be a response to the degradation of lymphatic vessels.[57]

4.3 Lymphedema staging

Lymphedema presents in three stages depending on the severity of congestion (see Table 1).

Table 1. Lymphedema staging by the International Society of Lymphology (ISL). [58]

Stage	**Symptoms**
Stage 0	Latent or prodromal stage. Lymphatic dysfunction can persist at this stage for several months or years before visible symptoms. There is no visible lymphedema or pitting, but lymph transport is impaired. Subjective symptoms may appear.
Stage I	Reversible lymphedema, with visible swelling and temporary pitting edema or collection of fluid. Fluid collection is protein rich and proliferating cell concentration can increase. Elevation of the affected area eases or completely reverts symptoms.
Stage II	Lymphedema with underlying tissue damage such as scarring, fibrotisation, and subcutaneous fat deposition, which are non-reversible. Visible swelling occurs with pitting. Pitting may finally disappear after extensive soft-tissue damage has taken place.
Stage III	Visible swelling and elephantiasis, deformation of affected area due to extensive fibrotisation and subcutaneous fat depositions. This stage involves severe soft-tissue damage and skin thickening occurs. Pitting can be resolved due to extensive tissue sclerosis.

Lymphedema has been defined as an interlimb volume difference of >200ml or >10%, difference between the affected and unaffected limb owing to lymph collection and/or fibrotisation.[59] Diagnosis also requires a complete patient history, charting symptoms and both imaging- and non-imaging -techniques to assess the severity and grade of lymphedema.[60] Techniques used to diagnose lymphedema include measuring bioelectrical impedance, perometry, circumference measurement and water displacement.[61] Multiple imaging techniques exist to visualize the physical changes present in lymphedema including lymphangiography, lymphoscintigraphy, and ultrasound, as well as contrast- and non-contrast -aided magnetic resonance imaging (MRI).[12]

4.4 Breast cancer-related lymphedema following damage to lymphatic tissue

Surgical approaches in breast cancer include tumour excision and either sentinel lymph node biopsy (SLNB) or axillary lymph node dissection (ALND). [62] In the case of breast cancer, the axillary lymph nodes are often affected due to breast lymph primary drainage to these lymph nodes. [63] SLNB is commonly performed to determine the spreading of cancer cells. 2-3 axillary lymph nodes are removed, and histological analysis of the sample is further examined to determine whether cancerous cells can be discerned. Further removal of axillary lymph nodes is called ALND, which is generally performed if cancerous cells are observed in several axillary sentinel nodes, amongst other criteria.[62]

The overall incidence of BCRL ranging from stage I-III in a five-year follow-up has been found to be 42%, with 89% of cases occurring during the first three years following surgery[6]. Incidence however varies and depends on the type of treatment received. [61] SLNB, ALND [61,64] and radiative treatments[7] heighten the risk of developing breast cancer-related lymphedema.

A comprehensive meta-analysis[64] conducted in 2013 found the incidence of lymphedema following ALND to be 19.9% and following SLNB to be 5.6%. Incidence of lymphedema appeared to increase up to 2 years after diagnosis or breast cancer surgery and when assessed by more than one diagnostic method, pooled incidence was up to 28.2%. Factors which increased risk of developing lymphedema were extensive surgery, such as mastectomy, axillary lymph node removal, a higher count of dissected lymph nodes as well as being overweight or obese. Another meta-analysis by Shaitelman et al.[61] (2015) found similar results, where incidence of secondary lymphedema following ALND was 22.3% and SLNB was 6.3%.

4.4.1 Secondary complications of chronic lymphedema

Chronic lymphedema can predispose patients to develop other secondary complications. A recent clinical study by Klüsch et al.[65] (2021) demonstrated that chronic lymphedema significantly correlates with increased intima media-thickness in both the brachial and radial arteries, therefore independently causing vascular wall remodeling. Chronic inflammatory

processes paired with impaired lymphatic clearance and consequent dysfunctional lipid reabsorption can all contribute towards an increased rate of atherosclerotic processes.

Additionally, BCRL can cause patients to be susceptible to developing infections of the associated limb, such as lymphangitis, cellulitis, or erysipelas,[66] which can further exacerbate fibrotisation through inflammatory processes. Post-mastectomy secondary lymphedema also heightens the risk of developing lymphangiosarcoma (Stewart-Treves -syndrome) in the affected area, which is a rare vascular tumour type with a poor prognosis.[67]

4.5 Cellular and molecular changes in the stromal vascular fraction in lymphedema using traditional cell analysis methods

As adipose tissue hypertrophy is a consequence of lymphedema, experimental settings have aimed to identify related pathophysiologic changes.[11] Adipose tissue contains adipocytes, vascular cells, immune cells, and adipose-derived stromal cells (ASCs). The stromal vascular fraction (SVF) however is composed of all other cells in adipose tissue, except adipose cells. Several studies have noted changes within SVF of subcutaneous adipose tissue in the presence of lymphedema. [68,69]

Adipose tissue hypertrophy has been partially associated with prolonged and significant lymphatic stasis, which results in a hypoxic environment.[68] The expansion of adipose tissue also results in further hypoxia, activating hypoxia inducible factor 1 (HIF-1), promoting fibrotic processes in adipose tissue. Classically activated (M1) macrophages and adipocytes in hypoxia release chemokines and cytokines and decrease proliferator-activated receptor-gamma (PPAR-gamma), inhibiting preadipocyte differentiation. Adipocytes and resident macrophages begin to produce collagen.[70] Increasing angiogenesis in rapidly hypertrophic adipose tissue has been shown to reduce fibrosis by lowering hypoxia.[71] Results indicate that by resolving lymphatic stasis induced hypoxia and immune cell response, adipose hypertrophy and consequent fibrotisation could be prevented.

Lymphedema adipose tissue in lower extremities has also been found to contain significantly less ASCs and an imbalance of alternatively activated (M2) macrophage downregulation and M1 upregulation in comparison to healthy controls, potentially leading to limitations in reparative and homeostatic functions in lymphedema adipose tissue.[68]

Aschen et al.[9] (2012) demonstrated an increase in proteins promoting adipose differentiation in lymphedema mouse tail models. Lymphatic stasis resulted in fibrosis and adipose tissue deposits within the affected tail. Adiponectin, PPAR-gamma and cytosine-cytosine-adenosine-adenosine-thymidine enhancer-binding-protein-alpha (CCAAT/EBP-alpha) expression increased with a direct positive correlation to the increase in lymph stasis. PPAR-gamma and adiponectin expression were upregulated in cells within new adipose tissue. [9]

A study by Zampell et al[72] (2012) utilizing multi-colour flow cytometry noted that lymphedema in mouse-tail models induces a mixed inflammatory cell response with increases in neutrophils, T-helper, T-regulatory, macrophage and dendritic cells. Following lymph node dissection, T-helper cell populations spiked after which macrophage and dendritic cell populations increased as well. Additionally, a connection was identified between CD4+ cell depletion, and a decrease in inflammation, fibrosis, and adipose tissue deposition. The depletion of CD4+ cells also led to an increase in lymphangiogenesis. Therefore CD4+ cells appear to hold an important role in moderating inflammatory responses during lymphedema, which partly lead to consequent chronic side effects of lymphedema.

A further study by Avraham et al.[73] (2013) using immunohistochemistry, flow cytometry, electrophoresis and ELISA also displayed CD4+ activation and T-helper 2 (Th2) cell differentiation in lymph stasis. CD4+ was found to upregulate fibrosis and lymphatic dysfunction. By blocking Th2 differentiation, fibrosis was decreased, and lymphatic function increased, independent of lympangiogenic growth factors. Fibrosis was found to be an independent factor causing lymphedema, whereas lymphatic stasis was associated with fibrosis of lymphatic capillaries, suggesting that both processes cyclically exacerbate lymphatic dysfunction.

Ghanta et al.[74] (2015) found that lymphedema significantly increased the macrophage population with a preference for M2 differentiation. The decrease of macrophages correlated with an increase in collagen deposits, fibrosis, and a rise in CD4+ cells, also positively

influencing Th2 differentiation. Vascular endothelial growth factor-C (VEGF-C) was decreased, and lymphatic drainage was impaired. Interestingly, overall inflammatory responses were not significantly changed, but T-cell infiltration increased significantly following macrophage depletion. This suggests that macrophages hold a central role in upkeeping lymphatic function, increase VEGF-C expression and modulating T-cell recruitment and differentiation. [74]

4.6 Current treatment methods of secondary lymphedema

To date, there does not exist a cure for lymphedema.[12] Compressive garments are widely used post-operatively to prophylactically prevent or decrease lymphedema. Manual lymph therapy, bandaging and pneumatic compression therapy can also be used in addition to compressive garments.[53] Compressive garments provide symptom relief, but do not prevent disease progression in all patient cases.[75] If conservative treatment fails to be effective and lymphedema continues to progress, the resulting hypertrophy, fibrosis, and adipose tissue requires surgical intervention.[76]

Surgery is usually considered in cases of chronic lymphedema, and conservative efforts, such as physiotherapy, have not been successful in resolving the issue.[66] Microsurgical interventions include lymphaticovenous anastomoses (LVA) and vascularized lymph node transfer (VLNT). LVA and VLNT are best suited for early-stage lymphedema patients, as lymphatic tissue and vessels are still functional and identifiable.[12] LVA and VLNT can be performed simultaneously to induce faster lymphedema alleviating effects.[77] VLNT can result in considerable long-term improvements or downstaging of lymphedema, as well as a notable decrease in related infectious diseases and need for physiotherapy.[66] Liposuction is mainly used in cases of chronic, late-stage lymphedema to improve of limb functionality.[12] Liposuction is always used in conjunction with compressive garments, as the continued and constant use of compressive garments is vital for patients in retaining primary surgical outcomes.[76,78,79] Although QoL and functionality of the affected limb is definitively improved and lymphatic flow can be increased by decreasing adipose mass, liposuction does not correct the underlying pathology related to lymphedema and as such is not curative.[80]

Combining micro- and macrosurgical treatment options in late-stage lymphedema seem advantageous, as there is an aim to also resolve weakened lymphatic function in addition to debulking alone. A study by Leppäpuska et al.[79] (2019) compared combined liposuction with lymph node transfer with lymph node transfer alone. Combining techniques significantly reduced the need for compression garment use, decreased erysipelas infections, increased lymphatic function, and decreased upper limb volume (87.7% decrease) compared to lymph node transfer alone (27.5% decrease).

All current treatments for lymphedema are therefore aimed at alleviating and controlling the symptoms related to the pathology. Conservative treatment is not enough to consistently prevent lymphedema progression in all patient groups[75] and while long-term surgical results are promising, all surgical interventions are nevertheless invasive procedures with surgery-associated risks. Additionally, microsurgical results have not been consistently satisfactory as compression garments must still be worn after the procedure, as is the case with liposuction, requiring compression garments daily to upkeep surgical results.[81] Combining liposuction with lymph node transfer has still not resolved fully the need to wear compression garments and resolve lymphedema in all patient groups.[79]

4.6.1 Pharmacotherapeutic approaches in lymphedema

Currently there do not exist FDA-approved pharmacotherapeutic treatments for lymphedema[13].[13] Additionally, there have been few systematic reviews regarding pharmacotherapeutic agents. A review by Forte et al.[82] (2019) included both clinical trials and experimental mouse model data. Pharmacotherapy agents with significant results mainly alleviated inflammation, decreasing edema, skin thickness and improving lymphangiogenesis. Oral ketoprofen [83], oral selenium [75,84,85] and topical tacrolimus [86] were found to decrease lymphedema, which were all well tolerated by patients.

VEGF-related treatments have been of natural interest as potential therapeutic targets in lymphedema, as VEGF-C and vascular endothelial growth factor-D (VEGF-D) promote lymphangiogenesis and are involved with proliferation and normal development of the lymphatic system. They are facilitated by common receptors VEGFR-2 and VEGFR-3.[87]

However, overtly high levels of VEGF[88] and VEGF-C[87] cause leaky and immature vessels to form. Angiopoietin-1 (Ang-1) and -2 (Ang-2) are respectively central in angiogenesis[89] and incidentally coexpression of Ang-1 with VEGF has resulted in leakage resistant vessels and proangiogenesis in mice models.[88] Therefore potential therapeutic targets regarding lymphedema have been identified in the combination of VEGF and angiopoietin.[87]

Inflammatory cytokines tumour necrosis factor-alpha (TNF-alpha)[90] and interleukin 1-beta (IL-1beta)[91] are also positively correlated in lymphangiogenesis. Tumour necrosis factor-alpha (TNF-alpha) and IL-1beta lymphangiogenesis is directly mediated by VEGF-C and VEGF-D. By blocking VEGF-C and -D lymphangiogenesis was inhibited in mouse models. Some macrophage populations recruited by IL-1beta induced cytokines were found to secrete VEGF-C, while neutrophils were not found to be involved in lymphangiogenesis.[91]

Concerns do exist regarding the use of VEGF-C and VEGF-D in relieving lymphedema following breast cancer treatments, as VEGF-C and VEGF-D are also often produced by tumour cells, as well as inflammatory cells within tumours. Tumor-related lymphangiogenesis promotes metastasis and therefore could potentially predispose patients to metastatic breast cancer.[92,93]

4.6.2 Combining microsurgery with growth factor therapy

Tervala et al.[94] (2015) studied the possibility of combining microsurgical techniques with growth factor therapy in a mouse model. The study hypothesis postulated, that combining these methods would increase lymphangiogenesis and aid in lymph node transplantation efficacy. Adenoviral gene transfer vectors carrying VEGF-C and VEGF-D were found to increase lymphangiogenesis, and VEGF-C improved vessel function. Both VEGFR-2 and -3 cooperation was required for lymph node survival, lymphangiogenesis and vessel functionality.

In a study by Lähteenvuo et al.[95] (2010), lymph node transfer was combined with a single injection of growth factors in a pig model. Stable lymphatic vasculature was formed in the transplanted lymph node particularly following the injection with VEGF-C. Therefore, a brief

treatment duration directly associated with lymph node transfer appears sufficient for stable lymphatic structures to form.

Hartiala et al.[96] (2020) commenced a clinical trial in breast cancer patients with upper limb lymphedema comparing single injection VEGF-C gene therapy combined with lymph node transfer, and lymph node transfer alone. VEGF-C was well tolerated with mild side effects. However, phase II results were inconclusive, as the baseline differences between control and treatment group were too significant, although volume reduction and QoL was improved in both groups. [97] No serious side effects were observed at a 24 month follow up.[98]

5. Lymphedema pathophysiology described at a cellular and molecular level using scRNA-seq

It is vital to gain detailed information about the pathophysiology of secondary lymphedema at a cellular and molecular level to find novel pharmacotherapeutic treatment or prevention methods in the future. Secondary lymphedema has been widely studied for this purpose, and important factors in cell level remodellation and reorganization have already been identified.[65,72–74] These have led to some advances in therapeutic approaches so far.[57,98]

However, it is evident that many treatment methods involving pharmacotherapeutics have been evaluated on animal models only [57,86,99], have been limited in clinical study size [83–85,100] or have lacked in conclusive results.[97] Anti-inflammatory pharmacotherapeutics have also not been found to reverse or ameliorate the damage to lymphatic vessels following lymphedema, therefore not resolving the underlying issue behind lymphedema.[101]

While traditional methods have been beneficial so far in understanding receptor and cell mechanics in secondary lymphedema, reasons to apply scRNA-seq in future are in better understanding genetic, proteomic and signaling changes in the lymphedema environment, to find alternative therapeutic targets and pathways to attenuate the condition.

5.1 Bulk RNA-sequencing and lymphedema pathophysiology

Bulk RNA-sequencing was used by Gousopoulos et al.[101] (2016) to identify changes in cell composition and gene regulation following surgically induced lymphedema in a mouse tail model. *Foxp3* gene was amongst the most upregulated genes in the study, and the expression is linked directly to regulatory T cells (Treg-cells). CD4+ depletion was shown to decrease lymphedema progression, and depletion of Treg-cells in turn caused an increase in lymphedema and T-helper 1 (Th1) and Th2 cells inflammatory response. Treg depletion led to expression of tumour growth factor-beta1 (TGF-beta1) and upregulation of cytokines *IL-4*, *IL-13* and *IFN-gamma*, leading to increased fibrosis and possible lymphatic dysfunction.

The expansion of Treg population again attenuated lymphedema development and reversed inflammatory response, fibrosis, and edema, thus identifying Treg-cells as a possible therapeutic target. Il2-c, which has been used in the mouse tail setting to expand the Treg-cell population, was however deemed partially unsafe as a clinical therapeutic, due to unknown potential side effects on effector T-cells. However, autologous or donor Treg-cells were identified as a safer immunotherapeutic strategy in a clinical setting, although risks involving cancer cell expansion or a decrease in anti-tumour immune response were also realized. Results following adoptive Treg-cell transfer indicated a marked decrease in fibrosis and inflammation, specifically decreasing levels of *TGF-beta1* and *IL-10*.[101]

5.2 Using scRNA-seq to map out pathophysiologic changes and cell interactions in lymphedema

To date, only two studies utilizing scRNA-seq to map out the pathophysiology of secondary lymphedema exists.[102,103] The advantage to scRNA-seq in this setting is the method’s potential to identify a vastly larger variety of possible related cells and markers in its pathophysiology, and to recognize more complex connections and pathways between said cells. Also, scRNA-seq allows for the identification of unexpected of previously unknown cell populations.

5.2.1 Single cell transcriptional profiling of adipose derived stromal cells in upper extremity lymphedema

Levi et al.[103] (2013) compared adipogenic gene signaling in ASCs between upper extremity chronic lymphedema patients (N = 3) and control samples (N = 3) using single cell transcriptional profiling. The stage of lymphedema and the primary cause of lymphedema in the cohort was not specified in the study. 48 genes were profiled from 200 human cells. Eleven differentially expressed genes (DEGs) with the highest correlation were identified between the lymphedema and control group used in clustering and assigning cell types.

ASCs in a lymphedema environment were found to contain markedly higher adipogenic potential and enhanced differentiation towards an adipogenic phenotype, driving the growth of adipose tissue in chronic lymphedema. All adipogenic genes were expressed significantly higher, including *LPL* and PPAR-gamma. One suggestion for the conversion through mechanical factors, for example increased hydrostatic pressure. The exact mechanism however, remained unclear. [103]

Single-cell transcriptional profiling was used to identify differences in genetic expression between control and lymphedema groups. Four distinct clusters were identified with common gene expression patterns. Individual cells were assigned to each cluster and cell proportions between the control and lymphedema groups had significant variation. Transcriptional profiles between the clusters also varied significantly, which would suggest that lymphedema causes changes in multiple signaling pathways in comparison to the healthy state. The gene *KLF4*, which has a role in stem cell induction [104], was downregulated in two distinct cell clusters.[103]

VEGF-A and VEGF-B expression was significantly reduced in lymphedema-associated ASCs in both normoxic and hypoxic conditions, demonstrating lower vasculogenity. Tubulus formation and angiogenic growth was significantly downregulated in ASC lymphedema samples. However, SLP-76 and Syk pathways -which play a role in regulating vasculogenesis and lymphangiogenesis[105]- displayed a higher protein expression in lymphedema samples, possibly suggesting compensative mechanisms or underlining dysfunctionality in vasculogenesis in lymphedema. Osteogenic differentiation was not significantly affected.[103]

5.2.2 *Single cell RNA sequencing of adipose tissue in lower extremity secondary lymphedema*

Liu et al.[102] (2022) sequenced 70,209 cells of the SVF of adipose tissues from both secondary lymphedema patients (N = 5) and healthy donors (N = 4). Subcutaneous adipose tissue samples were collected from five patients with stage III lower extremity lymphedema and the control group contained four healthy donors with no significant difference in BMI. Unbiased clustering resulted in 21 clusters, which were associated with 10 cell lineages. The ASC lineage contained 49,2% of SVF cells, and immune cells comprised 49,9% of the SVF, including lymphocytes and myeloid cells. Myeloid cells were mainly macrophages, but mast cells, dendritic cells (DC) and plasmacytoid dendritic cells were also present. Lymphocytes included T cells, B cells, natural killer (NK) cells and natural killer T (NKT) cells. Subpopulations of T cells included CD4+ helper T cells, regulatory T cells (Treg), CD8+ T cells, as well as proliferative and non-proliferative NKT cells. The vascular cells which were identified were pericytes and endothelial cells.

Comparing the expression of cells in the control group, relative proportions of cell populations were significantly altered. Especially lymphocyte lineages were increased (T-, NK- and NKT-cells), while myeloid lineages were decreased (macrophages, DCs). CD4+ T cells, ASCs, NK cells and NKT cells were significantly increased, whereas the macrophage subpopulation especially was decreased. The rise in lymphocyte lineages and decrease of myeloid lineages was accounted to be a result of adaptive immunity activation in chronic stages of lymphedema.[102]

Dysregulated pathways were also investigated using gene set enrichment analysis (GSEA). Extracellular matrix organization and collagen formation were significantly upregulated, which was concurrent with consequent fibrotisation processes during chronic lymphedema. Glycosylation pathways were also significantly upregulated, as well as DNA damage responses, indicating both potential pathways of chronic lymphedema as well as cellular responses to inflammation. ASC responses demonstrated a decrease in translation, energy metabolism and a response to endoplasmic reticulum stress. Interestingly, ASCs in advanced lymphedema demonstrated a significant decrease in adipogenesis, but an increase in

osteogenesis, reflecting dysregulation of cellular pathways at the advanced stage of lymphedema.[102]

Furthermore, the upregulation of certain genes was identified within associated cellular subpopulations. For example, the gene expression of *CLEC3B* was significantly higher in the lymphedema group, suggesting a central role in the progression of lymphedema and its potential role as for future target therapy. A knockdown of the *CLEC3B* gene was performed, and pathways and mRNA expression were compared between the control and study groups. Extracellular matrix pathways were downregulated in the knockdown group, and mRNA expression of genes associated with fibrosis (*COL1A1, CCN2, FN1*) were significantly downregulated, as was the marker for the ASC population *PRG4.*[102]

Macrophage population genotype and phenotype expression was investigated. In advanced lymphedema, proinflammatory macrophage subpopulations were upregulated, while anti-inflammatory tissue resident macrophage subpopulations were downregulated. This suggests that certain subpopulations of proinflammatory macrophages play a role in lymphedema and its proinflammatory microenvironment. The proinflammatory macrophage subpopulation strongly expressed *TREM1*, which is transcribed to a receptor serving to increase inflammatory response and engage myeloid cells into the site of inflammation [106]. As *TREM1* can be blocked using LR12 peptide, murine LR12 was used in a lymphedema mouse tail model to demonstrate the effects *in vivo*. mLR12 treated mice demonstrated a significant decrease in tail lymphedema, compared to the control group. Cd11b+Trem1+ cells were significantly decreased, and mousetail cross-section stains showed a marked decrease in fibrosis. Additionally, gene expression of proinflammatory cytokines such as *Il1b* and *Tnf* were decreased, as well as *CD68,* which is normally expressed in macrophage activation and inflammatory responses.[102]

Finally, cell-to-cell communication was also explored. In the healthy control group, ASCs and macrophages had the most interaction. In the lymphedema model, the most interactions were between vascular endothelial cells and ASCs. ASCs were also the primary source of macrophage colony stimulating factor 1 (*CSF1*), which is central to macrophage survival and *CSF1* expression was significantly increased in lymphedema. A connection has previously been established between increased platelet derived growth factor D (PDGFD) signaling and fibroblast differentiation and proliferation, and type I collagen secretion [107]. PDGFD-PGFDR

interactions were also significantly increased in the lymphedema model between vascular endothelial cells, NKT cells, pericytes and ASCs, underlining the significance of PDGFD in chronic lymphedema and fibrotisation. Additionally, chemokine signaling was altered and other specific changes in ligand-receptor interactions were identified in the lymphedema model.[102]

The study warrants further investigation into *TREM1*-suppressants such as LR12 as a possible therapeutic target in future treatment of lymphedema. Further investigation would also be recommended on the prevention of lymphedema also using *TREM1*-suppressants. Additionally, genetic suppression of the *CLEC3B* gene could alleviate lymphedema symptoms and would also be a point of interest in the future.[102]

6. Discussion: Information gained using scRNA-seq in chronic secondary lymphedema in comparison to traditional methods

Earlier studies using more conventional methods investigating pathophysiology have established a partial understanding behind the processes leading to secondary lymphedema, adipose hypertrophy, and fibrotic tissue formation.[9,72–74] scRNA-seq can be useful in understanding details related to the underlying pathophysiology, including cell-to-cell signaling, metabolic pathways, proteomic expression and altered cell composition in various environmental states.[5]

By investigating cell-signaling pathways, transcriptomes, genetic expression, cell subpopulations and associated receptors, novel information can be found regarding the progression and treatment of lymphedema, when compared to traditional cell analysis methods. Another key interest is in investigating temporal changes in cell and molecular structure during different stages of lymphedema.

6.1 Cell populations involved in lymphedema

Liu et al.[102] (2022) identified a relative, significant increase in CD4+ cells, as more conventional methods have previously described.[72,73] In the study, NK, NKT cells and

proinflammatory macrophage subpopulations were significantly increased, while the anti-inflammatory resident macrophage subpopulation was decreased. This was believed to be owing to an adaptive immune reaction in chronic lymphedema. A strong interaction between ASCs and macrophages was identified. ASCs were a primary source of *CSF1*, which macrophage populations are dependent on. Proinflammatory macrophages in chronic lymphedema expressed *TREM1*, transcribed to a receptor leading to recruitment of myeloid cells and an increase in inflammatory response at the site of inflammation. Inflammatory cytokines *Il1b* and *Tnf* were decreased following *TREM1* blockage.[102]

By gaining genetic and proteomic information through scRNA-seq data analytics, treatment options can be directed towards the downregulation of proinflammatory genes, and the progression of lymphedema could theoretically be attenuated or reversed. [72] Blocking *TREM1* -activation by mLR12 for example resulted in a significant decrease of lymphedema and fibrosis.[102]

6.2 Molecular changes in adipose tissue hypertrophy in lymphedema

Adipogenic signaling was highly upregulated in Levi et al.[103] (2013) scRNA-seq study. All adipogenic genes which were studied were expressed in significantly higher amounts, including *LPL* and PPAR-gamma. Conversely, Liu et al. (2022) found that chronic lymphedema resulted in a significant decrease in adipogenesis.[102]

Discrepancy between results could be explained by a different staging of lymphedema. It is possible, that the adipogenic potential of ASCs decreases as chronic lymphedema progresses and hypoxia increases, owing to tissue fibrotisation.

6.3 Involvement and population size of ASCs

Interestingly, in the study by Liu et al. [102] study (2022), ASCs were found to be increased in chronic stage III lymphedema. Following the knockdown of the *CLEC3B* gene, which was upregulated in lymphedema, was followed by a decrease in ASC population marker *PRG4*. This is paradoxical to earlier findings using traditional cell analysis, where ASCs in lymphedema adipose tissue have been significantly reduced[68]. Additionally, the scRNA-seq

study by Levi et al.[103] (2013) found that the stem-cell inducing gene *KLF4* was downregulated, which is more in line with previous results using traditional analytics and suggests a decrease in ASC induction.

The results seem to suggest a fluctuation of ASC involvement and population in varying conditions of lymphedema. It is possible, that ASC population size can differ, depending on the environmental conditions and staging, with a possible effect on the location of lymphedema.

6.4 Fibrotisation and dysregulated signaling and differentiation pathways

Chronic lymphedema using traditional cell analysis methods demonstrates a shift towards fibrotisation when lymph stasis is prolonged and inflammatory cells are recruited[73,101] and after adipose hypertrophy and hypoxia has increased,[70] while fibrosis independently increases lymphedema.[9,73] The involvement of CD4+ cells[72] and Th2[73] have been identified as central to fibrotisation processes.

In the Liu et al. (2022) study, using GSEA, ECM organization and collagen formation increased, which is in line with fibrotisation.[102] PDGF interactions, which are involved with type I collagen secretion and fibroblast differentiation and proliferation,[107] were significantly increased between several cell types, suggesting a mechanism behind fibrotisation of lymphedematous tissue. Instead, osteogenesis increased, implying dysregulation in metabolic pathways. Additionally, the knockdown of the *CLEC3B* gene which was upregulated in lymphedema, was followed by a decrease in several genes associated with fibrosis, and blocking *TREM1*, which is involved in myeloid cell engagement and inflammatory response, also led to a decrease in fibrosis.[102] Interestingly, Levi et al. (2013) did not find osteogenic differentiation to be significantly affected,[103] which is converse to the results in the Liu et al. (2022) study.[102]

This discrepancy between osteogenic differentiation may be owing to a difference between lymphedema staging or difference in the lymphedema timeframe. Regardless, there was clear evidence in both studies, that several signaling pathways were markedly dysregulated, suggesting that lymphedema generally leads to a state of disarray in signaling.[102,103]

6.5 VEGF and PDGF in lymphedema

The role of VEGF subtypes in lymphedema have been well described in several traditional studies, as VEGFs can increase lymphangiogenesis and neovasculature and promote resolution of lymphedema. [94–96,99] However, signaling patterns and vascular endothelial cell involvement have not previously been thoroughly mapped out.

In the study by Liu et al. (2022) vascular endothelial cell and ASC signaling increased the most, with significantly increased interactions between the PDGFD-PGDFR complex, relating to both fibrosis progression and ASC proliferation.[102] Interactions between VEGFs and VEGFR were not described, but there is an association between cross-family binding of PDGF and VEGFR2, suggesting a possible relationship between the growth factor-receptor pairs.[108] The study by Levi et al. (2013) demonstrated that VEGF-A and VEGF-B expression of ASCs in lymphedema was decreased in both normoxia and hypoxia.[103]

The increase of angiogenesis has been linked with a resolution of hypoxia induced adipose hypertrophy and fibrosis,[71] which is in line with the scRNA-seq results.

6.6 Concluding remarks and synopsis

The studies by Levi et al.[103] (2013) and Liu et al.[102] (2022) demonstrate how scRNA-seq can be used to gain further insight into cell signaling, genetic composition, transcriptional activity, and protein expression in a lymphedematous environment. While results varied regarding ASC involvement, osteogenic differentiation and adipogenicity, these discrepancies underline the complexity of lymphedema as a pathological continuum.

Compared to traditional cell analytics, scRNA-seq can add to previously gained information such as identifying genetic markers like *TREM1,* associated with proinflammatory macrophages involved in myeloid recruitment and inflammatory response. Other central genes involved in adipogenesis were identified such as *LPL* and PPAR-gamma[103] as well as *CLEC3B*, which is associated with the upregulation of fibrosis in chronic lymphedema.[102] Inhibiting specific proinflammatory and profibrotic genes could provide a target in future lymphedema care.

scRNA-seq can also provide an explanation for the reason why VEGF- and other neovasculature promoting treatments have shown potential in treating lymphedema,[94–96] as ASCs have decreased expression of VEGF-A and VEGF-B, although the expression of VEGF-C -a commonly studied and used therapeutic in lymphedema- was not explored in the study [103]. Additionally, PDGFD and PDGFR interactions were identified to be central relating to fibrosis.[102] Understanding the interactions and pathways behind the physiological manifestations of lymphedema is vital regarding pharmacotherapeutic development.

7. Materials and methods

This thesis was conducted as a literature review. Most of the sources used in this review were searched utilizing the PubMed and Scopus databases, with a focus on primary sources. Meta-analyses and systematic reviews were also used primarily regarding background information. Additionally, a few government-moderated healthcare and pharmacotherapeutic websites were used for background information. Search terms included variations of scRNA-seq and lymphedema, or terms relating to adipose hypertrophy and fibrosis. Lymphedema searches also included terms involving breast cancer treatments such as conservative, surgical and pharmacotherapeutic approaches, and variations of lymphadenectomy and axillary lymph node dissections.

8. Strengths and limitations

Limitations of this literature review owe to the small number of primary sources regarding scRNA-seq in a chronic lymphedema setting. The study by Liu et al. (2022) used as a reference for scRNA-seq in patients with chronic secondary lymphedema was conducted on a limited number of patients (control N = 4, case N = 5) and the study was conducted on chronic stage III lower limb lymphedema following cervical cancer treatments, not on post-treatment breast cancer upper limb secondary lymphedema patients. Additionally, part of the study was conducted on a mouse-tail model.[102] The second primary source of this review by Levi et al. (2013) used as a reference for scRNA-seq in chronic lymphedema was again conducted on a limited number of patients (control N = 3, case N = 3) with upper extremity lymphedema, but the stage and cause of lymphedema was not specified.[103] The scope of the study was mainly

focused on ASC transcriptional profiling and studying vascularity and osteogenic differentiation capacity. As the study was conducted at the early stages of scRNA-seq development, data analysis software and databases used in modern scRNA-seq were not yet available or were provided with more limited options.

Therefore, direct conclusions cannot be drawn using these results gained by scRNA-seq, when aiming to focus on post-treatment breast cancer related upper extremity secondary lymphedema. Furthermore, the studies which were compared to the results in the scRNA-seq study conducted by traditional cellular and molecular analysis methods were not standardized or limited to a single type or stage of lymphedema, and several studies regarding molecular and cellular changes in lymphedema were conducted on animal-models instead of humans.[94,95,99]

However, each step towards uncovering secondary lymphedema development and the expression of genetic and molecular markers or other functions of dysregulation help future endeavors to find targeted care options. It is clear, that scRNA-seq can provide information that has not been available using earlier cell analysis methods. Results imply, that scRNA-seq can also solidify and enhance previously attained results. Optimally scRNA-seq can bridge the gap between molecular level changes and macroscopic effects as seen in patient phenotypes and provide variance of pathophysiological information on an individualized level. Later studies would benefit from scRNA-seq from samples directly from post-treatment breast cancer related lymphedema taken from a larger cohort of patients with a range of lymphedema stages, comparing the results to age-, lifestyle-, physiology- and BMI- matched controls.

9. Conclusions

Using scRNA-seq to understand the pathophysiology behind chronic secondary lymphedema to identify potential therapeutic targets appears promising. Regarding the etiology behind breast cancer related post-treatment secondary lymphedema, the mechanical injury to axillary lymph nodes and lymphatic vessels caused either by ALND or SLNB and possible radiative or chemotherapy seem to play a pivotal role in the initiation of the pathological state.[7] As such, it could be argued that pharmacotherapeutic agents alone are not enough to yield curative results in such cases of secondary lymphedema and surgical approaches hold an important role both in the present and future.

However, as the development of secondary lymphedema on a cellular and molecular level has been incompletely understood,[11] pharmacotherapeutic trials so far have been less focused on targeted approaches. The prevention and treatment of secondary lymphedema warrants further investigation into therapeutic possibilities as auxiliary treatment methods. New methods could hold potential in decreasing the severity and progression of lymphedema, boosting post-operative recuperation and lymphatic drainage and neogenesis of lymphatic vessels. Injectable viral vectors,[96] direct contact release gels containing the therapeutic of choice,[99] and specified antibodies[109] are a few possible ways to deliver targeted care to the lymphedema area.

scRNA-seq can be effectively used in specifying new targets for future care in secondary lymphedema. This can be achieved by detailed molecular mapping of the lymphedema process. By mapping cell-to-cell signaling, clustering associated cell types, identifying up- and downregulation of related genes and mapping the transcriptome at different stages and time frames of lymphedema could provide insight into possible ways to prevent or alleviate lymphedema, fibrotic processes, and adipose tissue hypertrophy. Another area of interest would be in recognizing genetic or molecular risk factors, or other intracellular processes or markers, which precede severe lymphedema or lymphedema progression.

Further investigations into scRNA-seq and secondary lymphedema would benefit from including a larger study cohort and a comparison of molecular and cellular changes at different stages of lymphedema. In the case of breast cancer patients, a further study would be recommended to be conducted on upper limb chronic secondary post-treatment lymphedema in the future.

Other ways to expand upon the research would be to utilize other NGS techniques such as snRNA-seq and patch-seq to compare different datasets and results. Changing QC metrics can also result in different results post-processing,[34] as can the choice of sequencing and amplification system.[2] When clustering datasets, different batch effect correction methods[46] can also be utilized to better visualize differences between datasets from different points of time, or different stages of pathology.

Temporal and spatial cell analysis using scRNA-seq is currently an emerging field.[110] scRNA-seq can also be combined with other genetic tools and protocols, whether in screening applications or gene modification such as using CRISPR.[111] Although currently the primary application of scRNA-seq lies in understanding cell functions and pathophysiology of diseases, future applications are promising for the use of scRNA-seq in personalized medicine and clinical diagnostics.[110]

As the technique is relatively new, there is an immense amount of research yet to be expanded upon and methodology for scRNA-seq is constantly improved, yielding results with even more detail and specificity. Additionally, the programs and algorithms used in data analysis, quality control and quantification of data are also constantly built upon and reiterated, allowing for better understanding the data at hand. scRNA-seq transcriptomic open-access libraries are expanded continuously. Although the golden standard for scRNA-seq has not yet been established owing to the rapid development of new techniques and alternate strategies in data extraction and analysis, further research and time will undoubtedly allow for a comparative analysis of different methods in the future, making scRNA-seq more robust, reliable, and replicable. NGS techniques and scRNA-seq demonstrate great potential for a wide array of uses in many areas of healthcare in the future.

10. Bibliography

1. Kukurba KR, Montgomery SB. RNA Sequencing and Analysis. *Cold Spring Harb Protoc*. 2015;2015(11):951. doi:10.1101/PDB.TOP084970
2. See P, Lum J, Chen J, Ginhoux F. A single-cell sequencing guide for immunologists. *Front Immunol*. 2018;9(OCT):415498. doi:10.3389/FIMMU.2018.02425/BIBTEX
3. Tang F, Barbacioru C, Wang Y, et al. mRNA-Seq whole-transcriptome analysis of a single cell. *Nat Methods*. 2009;6(5):377-382. doi:10.1038/NMETH.1315
4. Lexogen. Classical Single-Cell RNA Sequencing: a comprehensive overview | Lexogen. Published January 3, 2024. Accessed February 28, 2024. https://www.lexogen.com/classical-single-cell-rna-sequencing-a-comprehensive-overview/
5. Haque A, Engel J, Teichmann SA, Lönnberg T. A practical guide to single-cell RNA-sequencing for biomedical research and clinical applications. *Genome Med*. 2017;9(1):1-12. doi:10.1186/S13073-017-0467-4/TABLES/1
6. Norman SA, Localio AR, Potashnik SL, et al. Lymphedema in Breast Cancer Survivors: Incidence, Degree, Time Course, Treatment, and Symptoms. *Journal of Clinical Oncology*. 2009;27(3):390. doi:10.1200/JCO.2008.17.9291
7. Kim M, Kim SW, Lee SU, et al. A model to estimate the risk of breast cancer-related lymphedema: combinations of treatment-related factors of the number of dissected axillary nodes, adjuvant chemotherapy, and radiation therapy. *Int J Radiat Oncol Biol Phys*. 2013;86(3):498-503. doi:10.1016/J.IJROBP.2013.02.018
8. Kataru RP, Wiser I, Baik JE, et al. Fibrosis and secondary lymphedema: chicken or egg? *Transl Res*. 2019;209:68. doi:10.1016/J.TRSL.2019.04.001
9. Aschen S, Zampell JC, Elhadad S, Weitman E, De Brot M, Mehrara BJ. Regulation of adipogenesis by lymphatic fluid stasis: Part II. Expression of adipose differentiation genes. *Plast Reconstr Surg*. 2012;129(4):838-847. doi:10.1097/PRS.0B013E3182450B47
10. Penha TRL, Botter B, Heuts EM, Voogd AC, Von Meyenfeldt MF, Van Der Hulst RR. Quality of Life in Patients with Breast Cancer-Related Lymphedema and

Reconstructive Breast Surgery. *J Reconstr Microsurg*. 2016;32(6):484-490. doi:10.1055/S-0036-1572538/ID/JR150189-31

11. Azhar SH, Lim HY, Tan BK, Angeli V. The Unresolved Pathophysiology of Lymphedema. *Front Physiol*. 2020;11. doi:10.3389/FPHYS.2020.00137
12. Salehi BP, Sibley RC, Friedman R, et al. MRI of Lymphedema. *J Magn Reson Imaging*. 2023;57(4):977. doi:10.1002/JMRI.28496
13. Pal S, Rahman J, Mu S, Rusch NJ, Stolarz AJ. Drug-Related Lymphedema: Mysteries, Mechanisms, and Potential Therapies. *Front Pharmacol*. 2022;13. doi:10.3389/FPHAR.2022.850586
14. Jovic D, Liang X, Zeng H, Lin L, Xu F, Luo Y. Single-cell RNA sequencing technologies and applications: A brief overview. *Clin Transl Med*. 2022;12(3). doi:10.1002/CTM2.694
15. Lambrechts D, Wauters E, Boeckx B, et al. Phenotype molding of stromal cells in the lung tumor microenvironment. *Nat Med*. 2018;24(8):1277-1289. doi:10.1038/S41591-018-0096-5
16. Chung W, Eum HH, Lee HO, et al. Single-cell RNA-seq enables comprehensive tumour and immune cell profiling in primary breast cancer. *Nat Commun*. 2017;8. doi:10.1038/NCOMMS15081
17. Pan Y, Lu F, Fei Q, et al. Single-cell RNA sequencing reveals compartmental remodeling of tumor-infiltrating immune cells induced by anti-CD47 targeting in pancreatic cancer. *J Hematol Oncol*. 2019;12(1). doi:10.1186/S13045-019-0822-6
18. Yu X, Chen YA, Conejo-Garcia JR, Chung CH, Wang X. Estimation of immune cell content in tumor using single-cell RNA-seq reference data. *BMC Cancer*. 2019;19(1). doi:10.1186/S12885-019-5927-3
19. Nguyen A, Khoo WH, Moran I, Croucher PI, Phan TG. Single cell RNA sequencing of rare immune cell populations. *Front Immunol*. 2018;9(JUL):379154. doi:10.3389/FIMMU.2018.01553/BIBTEX
20. Hu P, Zhang W, Xin H, Deng G. Single cell isolation and analysis. *Front Cell Dev Biol*. 2016;4(OCT):116. doi:10.3389/FCELL.2016.00116/BIBTEX
21. Kivioja T, Vähärautio A, Karlsson K, et al. Counting absolute numbers of molecules using unique molecular identifiers. *Nature Methods 2011 9:1*. 2011;9(1):72-74. doi:10.1038/nmeth.1778

22. Islam S, Zeisel A, Joost S, et al. Quantitative single-cell RNA-seq with unique molecular identifiers. *Nature Methods 2013 11:2*. 2013;11(2):163-166. doi:10.1038/nmeth.2772
23. Ziegenhain C, Hendriks GJ, Hagemann-Jensen M, Sandberg R. Molecular spikes: a gold standard for single-cell RNA counting. *Nature Methods 2022 19:5*. 2022;19(5):560-566. doi:10.1038/s41592-022-01446-x
24. Fu Y, Wu PH, Beane T, Zamore PD, Weng Z. Elimination of PCR duplicates in RNA-seq and small RNA-seq using unique molecular identifiers. *BMC Genomics*. 2018;19(1). doi:10.1186/S12864-018-4933-1
25. Marquina-Sanchez B, Fortelny N, Farlik M, et al. Single-cell RNA-seq with spike-in cells enables accurate quantification of cell-specific drug effects in pancreatic islets. *Genome Biol*. 2020;21(1):1-22. doi:10.1186/S13059-020-02006-2/FIGURES/6
26. Kim JMH, Camarena A, Walker C, et al. Robust RNA-Seq of aRNA-amplified single cell material collected by patch clamp. *Sci Rep*. 2020;10(1). doi:10.1038/S41598-020-58715-Y
27. Yijyechern. Single-cell RNA sequencing workflow. Published online March 7, 2014.
28. Datlinger P, Rendeiro AF, Boenke T, et al. Ultra-high-throughput single-cell RNA sequencing and perturbation screening with combinatorial fluidic indexing. *Nature Methods 2021 18:6*. 2021;18(6):635-642. doi:10.1038/s41592-021-01153-z
29. Zeng W, Jiang S, Kong X, et al. Single-nucleus RNA-seq of differentiating human myoblasts reveals the extent of fate heterogeneity. *Nucleic Acids Res*. 2016;44(21):e158. doi:10.1093/NAR/GKW739
30. Rosenberg AB, Roco CM, Muscat RA, et al. SPLiT-seq reveals cell types and lineages in the developing brain and spinal cord. *Science*. 2018;360(6385):176. doi:10.1126/SCIENCE.AAM8999
31. Zheng GXY, Terry JM, Belgrader P, et al. Massively parallel digital transcriptional profiling of single cells. *Nature Communications 2017 8:1*. 2017;8(1):1-12. doi:10.1038/ncomms14049
32. Rousselle T V, Mcdaniels JM, Shetty AC, Bardhi E, Maluf DG, Mas VR. An optimized protocol for single nuclei isolation from clinical biopsies for RNA-seq. Published online 123AD. doi:10.1038/s41598-022-14099-9

33. Smolander J. Cell Type Identification, Differential Expression Analysis and Trajectory Inference in Single-Cell Transcriptomics. Published online August 31, 2023. Accessed September 12, 2023. https://urn.fi/URN:ISBN:978-951-29-9393-2
34. Single-cell RNA-seq: Quality Control Analysis | Introduction to Single-cell RNA-seq - ARCHIVED. Accessed September 15, 2023. https://hbctraining.github.io/scRNA-seq/lessons/04_SC_quality_control.html
35. Vallejos CA, Risso D, Scialdone A, Dudoit S, Marioni JC. Normalizing single-cell RNA sequencing data: Challenges and opportunities. *Nat Methods*. 2017;14(6):565. doi:10.1038/NMETH.4292
36. Hwang B, Lee JH, Bang D. Single-cell RNA sequencing technologies and bioinformatics pipelines. *Experimental & Molecular Medicine 2018 50:8*. 2018;50(8):1-14. doi:10.1038/s12276-018-0071-8
37. Haghverdi L, Lun ATL, Morgan MD, Marioni JC. Batch effects in single-cell RNA-sequencing data are corrected by matching mutual nearest neighbors. *Nature Biotechnology 2018 36:5*. 2018;36(5):421-427. doi:10.1038/nbt.4091
38. Chazarra-Gil R, Van Dongen S, Kiselev VY, Hemberg M. Flexible comparison of batch correction methods for single-cell RNA-seq using BatchBench. *Nucleic Acids Res*. 2021;49(7):e42-e42. doi:10.1093/NAR/GKAB004
39. Tran HTN, Ang KS, Chevrier M, et al. A benchmark of batch-effect correction methods for single-cell RNA sequencing data. *Genome Biol*. 2020;21(1):1-32. doi:10.1186/S13059-019-1850-9/TABLES/1
40. Tran HTN, Ang KS, Chevrier M, et al. A benchmark of batch-effect correction methods for single-cell RNA sequencing data. *Genome Biol*. 2020;21(1):1-32. doi:10.1186/S13059-019-1850-9/TABLES/1
41. An S, Shi J, Liu R, et al. A Joint Batch Correction and Adaptive Clustering Method of Single-Cell Transcriptomic Data. *Mathematics*. 2023;11(24):4901. doi:10.3390/MATH11244901/S1
42. Townes FW, Hicks SC, Aryee MJ, Irizarry RA. Feature selection and dimension reduction for single-cell RNA-Seq based on a multinomial model. *Genome Biol*. 2019;20(1):1-16. doi:10.1186/S13059-019-1861-6/FIGURES/5
43. Cole MB, Risso D, Wagner A, et al. Performance Assessment and Selection of Normalization Procedures for Single-Cell RNA-Seq. *Cell Syst*. 2019;8(4):315-328.e8. doi:10.1016/J.CELS.2019.03.010

44. Rosenberg AB, Roco CM, Muscat RA, et al. Single-cell profiling of the developing mouse brain and spinal cord with split-pool barcoding. *Science*. 2018;360(6385):176-182. doi:10.1126/SCIENCE.AAM8999

45. Sato K, Tsuyuzaki K, Shimizu K, Nikaido I. CellFishing.jl: An ultrafast and scalable cell search method for single-cell RNA sequencing. *Genome Biol*. 2019;20(1):1-23. doi:10.1186/S13059-019-1639-X/FIGURES/11

46. Büttner M, Miao Z, Wolf FA, Teichmann SA, Theis FJ. A test metric for assessing single-cell RNA-seq batch correction. *Nat Methods*. 2019;16(1):43-49. doi:10.1038/S41592-018-0254-1

47. Tsuyuzaki K, Sato H, Sato K, Nikaido I. Benchmarking principal component analysis for large-scale single-cell RNA-sequencing. doi:10.1186/s13059-019-1900-3

48. Ianevski A, Giri AK, Aittokallio T. Fully-automated and ultra-fast cell-type identification using specific marker combinations from single-cell transcriptomic data. *Nat Commun*. 2022;13(1). doi:10.1038/S41467-022-28803-W

49. Efremova M, Vento-Tormo M, Teichmann SA, Vento-Tormo R. CellPhoneDB: inferring cell–cell communication from combined expression of multi-subunit ligand–receptor complexes. *Nature Protocols 2020 15:4*. 2020;15(4):1484-1506. doi:10.1038/s41596-020-0292-x

50. Armer J, Cheville A, Miller LT, Schmitz K, Stout N. Lymphedema: Symptoms, Treatment, Risk Factors, and More. Published July 30, 2022. Accessed April 25, 2023. https://www.breastcancer.org/treatment-side-effects/lymphedema

51. Breslin JW, Yang Y, Scallan JP, Sweat RS, Adderley SP, Murfee WL. Lymphatic Vessel Network Structure and Physiology. *Compr Physiol*. 2018;9(1):207. doi:10.1002/CPHY.C180015

52. Ridner SH. Breast Cancer Lymphedema: Pathophysiology and Risk Reduction Guidelines. *Oncol Nurs Forum*. 2002;29(9):1285-1293. doi:10.1188/02.ONF.1285-1293

53. Warren AG, Brorson H, Borud LJ, Slavin SA. Lymphedema: A comprehensive review. *Ann Plast Surg*. 2007;59(4):464-472. doi:10.1097/01.SAP.0000257149.42922.7E

54. Greene AK, Goss JA. Lymphedema Management: Diagnosis and Staging of Lymphedema. *Semin Plast Surg*. 2018;32(1):12. doi:10.1055/S-0038-1635117

55. Sleigh BC, Manna B. Lymphedema - StatPearls - NCBI Bookshelf. Published April 19, 2023. Accessed March 8, 2024. https://www.ncbi.nlm.nih.gov/books/NBK537239/#:~:text=Signs%20and%20symptoms%20of%20lymphedema,and%20tissue%20changes%3B%20skin%20discoloration%3B
56. Mihara M, Hara H, Hayashi Y, et al. Pathological Steps of Cancer-Related Lymphedema: Histological Changes in the Collecting Lymphatic Vessels after Lymphadenectomy. *PLoS One*. 2012;7(7):e41126. doi:10.1371/JOURNAL.PONE.0041126
57. Nakamura K, Radhakrishnan K, Wong YM, Rockson SG. Anti-Inflammatory Pharmacotherapy with Ketoprofen Ameliorates Experimental Lymphatic Vascular Insufficiency in Mice. *PLoS One*. 2009;4(12):e8380. doi:10.1371/JOURNAL.PONE.0008380
58. International Society of Lymphology. The Diagnosis and Treatment of Peripheral Lymphedema: 2020 Consensus Document of the International Society of Lymphology. *Lymphology*. 2020;53(1):3-19. doi:10.2458/lymph.4649
59. Armer JM, Stewart BR. A Comparison of Four Diagnostic Criteria for Lymphedema in a Post-Breast Cancer Population. *https://home.liebertpub.com/lrb*. 2005;3(4):208-217. doi:10.1089/LRB.2005.3.208
60. Kayiran O, De La Cruz C, Tane K, Soran A. Lymphedema: From diagnosis to treatment. *Turk J Surg*. 2017;33(2):51. doi:10.5152/TURKJSURG.2017.3870
61. Simona F. Shaitelman, Kate D. Cromwell, John C. Rasmussen, et al. Recent Progress in the Treatment and Prevention of Cancer-Related Lymphedema. *CA Cancer J Clin*. 2015;65(1):55-81. doi:10.3322/caac.21253
62. Toomey AE, Lewis CR. Axillary Lymphadenectomy. *Operative Strategy in General Surgery*. Published online July 24, 2023:491-496. doi:10.1007/978-1-4757-4172-8_47
63. Chatterjee A, Serniak N, Czerniecki BJ. Sentinel Lymph Node Biopsy in Breast Cancer: A Work in Progress. *Cancer J*. 2015;21(1):7. doi:10.1097/PPO.0000000000000090
64. DiSipio T, Rye S, Newman B, Hayes S. Incidence of unilateral arm lymphoedema after breast cancer: a systematic review and meta-analysis. *Lancet Oncol*. 2013;14(6):500-515. doi:10.1016/S1470-2045(13)70076-7

65. Klüsch V, Boyle EC, Rustum S, et al. Chronic unilateral arm lymphedema correlates with increased intima-media thickness in the brachial artery. *https://doi.org/101024/0301-1526/a000982*. 2021;51(1):19-23. doi:10.1024/0301-1526/A000982
66. Becker C, Assouad J, Riquet M, Hidden G. Postmastectomy Lymphedema: Long-term Results Following Microsurgical Lymph Node Transplantation. *Ann Surg*. 2006;243(3):313. doi:10.1097/01.SLA.0000201258.10304.16
67. Rivere AE, Klimberg VS. Lymphedema in the Postmastectomy Patient: Pathophysiology, Prevention, and Management. *The Breast: Comprehensive Management of Benign and Malignant Diseases*. Published online January 1, 2018:514-530.e4. doi:10.1016/B978-0-323-35955-9.00036-2
68. Tashiro K, Feng J, Wu SH, et al. Pathological changes of adipose tissue in secondary lymphoedema. *British Journal of Dermatology*. 2017;177(1):158-167. doi:10.1111/BJD.15238
69. Zampell JC, Aschen S, Weitman ES, et al. Regulation of Adipogenesis by Lymphatic Fluid Stasis Part I: Adipogenesis, Fibrosis, and Inflammation. *Plast Reconstr Surg*. 2012;129(4):825. doi:10.1097/PRS.0B013E3182450B2D
70. Buechler C, Krautbauer S, Eisinger K. Adipose tissue fibrosis. *World J Diabetes*. 2015;6(4):548. doi:10.4239/WJD.V6.I4.548
71. Michailidou Z, Turban S, Miller E, et al. Increased angiogenesis protects against adipose hypoxia and fibrosis in metabolic disease-resistant 11β-hydroxysteroid dehydrogenase type 1 (HSD1)-deficient mice. *J Biol Chem*. 2012;287(6):4188-4197. doi:10.1074/JBC.M111.259325
72. Zampell JC, Yan A, Elhadad S, Avraham T, Weitman E, Mehrara BJ. CD4+ Cells Regulate Fibrosis and Lymphangiogenesis in Response to Lymphatic Fluid Stasis. *PLoS One*. 2012;7(11):e49940. doi:10.1371/JOURNAL.PONE.0049940
73. Avraham T, Zampell JC, Yan A, et al. Th2 differentiation is necessary for soft tissue fibrosis and lymphatic dysfunction resulting from lymphedema. *FASEB Journal*. 2013;27(3):1114-1126. doi:10.1096/FJ.12-222695
74. Ghanta S, Cuzzone DA, Torrisi JS, et al. Regulation of inflammation and fibrosis by macrophages in lymphedema. *Am J Physiol Heart Circ Physiol*. 2015;308(9):H1065. doi:10.1152/AJPHEART.00598.2014

75. Kasseroller RG, Schrauzer GN. Treatment of secondary lymphedema of the arm with physical decongestive therapy and sodium selenite: a review. *Am J Ther*. 2000;7(4):273-279. doi:10.1097/00045391-200007040-00008
76. Brorson H, Svensson H. Liposuction and Controlled Compression Therapy in the Treatment of Arm Lymphedema following Breast Cancer. *Lund University Dissertations; (1998)*. Published online 1998. Accessed December 1, 2023. http://lup.lub.lu.se/record/38993
77. Chung JH, Hwang YJ, Park SH, Yoon ES. Preliminary outcomes of combined surgical approach for lower extremity lymphedema: supraclavicular lymph node transfer and lymphaticovenular anastomosis. *J Plast Surg Hand Surg*. 2022;56(5):261-269. doi:10.1080/2000656X.2021.1964980
78. Schaverien M V., Munnoch DA, Brorson H. Lymphedema Management: Liposuction Treatment of Lymphedema. *Semin Plast Surg*. 2018;32(1):42. doi:10.1055/S-0038-1635116
79. Leppäpuska IM, Suominen E, Viitanen T, et al. Combined Surgical Treatment for Chronic Upper Extremity Lymphedema Patients: Simultaneous Lymph Node Transfer and Liposuction. *Ann Plast Surg*. 2019;83(3):308-317. doi:10.1097/SAP.0000000000001828
80. Boyages J, Kastanias K, Health Bs, et al. Liposuction for Advanced Lymphedema: A Multidisciplinary Approach for Complete Reduction of Arm and Leg Swelling. *Ann Surg Oncol*. 2015;22:1263-1270. doi:10.1245/s10434-015-4700-3
81. Brorson H. Liposuction in Arm Lymphedema Treatment. *Scandinavian Journal of Surgery*. 2003;92:287-295.
82. Forte AJ, Boczar D, Huayllani MT, Lu X, McLaughlin SA. Pharmacotherapy Agents in Lymphedema Treatment: A Systematic Review. *Cureus*. Published online December 5, 2019. doi:10.7759/CUREUS.6300
83. Rockson SG, Tian W, Jiang X, et al. Pilot studies demonstrate the potential benefits of antiinflammatory therapy in human lymphedema. *JCI Insight*. 2018;3(20). doi:10.1172/JCI.INSIGHT.123775
84. Micke O, Bruns F, Mücke R, et al. Selenium in the treatment of radiation-associated secondary lymphedema. *Int J Radiat Oncol Biol Phys*. 2003;56(1):40-49. doi:10.1016/S0360-3016(02)04390-0

85. Zimmermann T, Leonhardt H, Kersting S, Albrecht S, Range U, Eckelt U. Reduction of postoperative lymphedema after oral tumor surgery with sodium selenite. *Biol Trace Elem Res*. 2005;106(3):193-203. doi:10.1385/BTER:106:3:193/METRICS
86. Gardenier JC, Kataru RP, Hespe GE, et al. Topical tacrolimus for the treatment of secondary lymphedema. *Nature Communications 2017 8:1*. 2017;8(1):1-11. doi:10.1038/ncomms14345
87. Saaristo A, Karkkainen MJ, Alitalo K. Insights into the Molecular Pathogenesis and Targeted Treatment of Lymphedema. *Ann N Y Acad Sci*. 2002;979(1):94-110. doi:10.1111/J.1749-6632.2002.TB04871.X
88. Thurston G, Suri C, Smith K, et al. Leakage-resistant blood vessels in mice transgenically overexpressing angiopoietin-1. *Science (1979)*. 1999;286(5449):2511-2514. doi:10.1126/SCIENCE.286.5449.2511/SUPPL_FILE/1043334C2.GIF
89. Hanahan D. Signaling vascular morphogenesis and maintenance. *Science (1979)*. 1997;277(5322):48-50. doi:10.1126/SCIENCE.277.5322.48/ASSET/AB006A18-F837-48DA-9191-2518BBC00DB6/ASSETS/GRAPHIC/48-2.GIF
90. Baluk P, Yao LC, Feng J, et al. TNF-alpha drives remodeling of blood vessels and lymphatics in sustained airway inflammation in mice. *J Clin Invest*. 2009;119(10):2954-2964. doi:10.1172/JCI37626
91. Baluk P, Hogmalm A, Bry M, Alitalo K, Bry K, McDonald DM. Transgenic overexpression of interleukin-1β induces persistent lymphangiogenesis but not angiogenesis in mouse airways. *Am J Pathol*. 2013;182(4):1434-1447. doi:10.1016/J.AJPATH.2012.12.003
92. Zheng W, Aspelund A, Alitalo K. Lymphangiogenic factors, mechanisms, and applications. *J Clin Invest*. 2014;124(3):878. doi:10.1172/JCI71603
93. Holopainen T, Bry M, Alitalo K, Saaristo A. Perspectives on lymphangiogenesis and angiogenesis in cancer. *J Surg Oncol*. 2011;103(6):484-488. doi:10.1002/JSO.21808
94. Tervala T V., Hartiala P, Tammela T, et al. Growth factor therapy and lymph node graft for lymphedema. *Journal of Surgical Research*. 2015;196(1):200-207. doi:10.1016/J.JSS.2015.02.031

95. Lähteenvuo M, Honkonen K, Tervala T, et al. Growth Factor Therapy and Autologous Lymph Node Transfer in Lymphedema. Published online 2011. doi:10.1161/CIRCULATIONAHA.110.965384
96. Hartiala P, Suominen S, Suominen E, et al. Phase 1 Lymfactin® Study: Short-term Safety of Combined Adenoviral VEGF-C and Lymph Node Transfer Treatment for Upper Extremity Lymphedema. *Journal of Plastic, Reconstructive & Aesthetic Surgery*. 2020;73(9):1612-1621. doi:10.1016/J.BJPS.2020.05.009
97. Herantis Announces Inconclusive Results from Phase II Study with Lymfactin in Breast Cancer Related Lymphedema ® - Herantis Pharma. Accessed January 11, 2024. https://herantis.com/mfn_news/herantis-announces-inconclusive-results-from-phase-ii-study-with-lymfactin-in-breast-cancer-related-lymphedema/
98. Leppäpuska IM, Hartiala P, Suominen S, et al. Phase 1 Lymfactin® Study: 24-month Efficacy and Safety Results of Combined Adenoviral VEGF-C and Lymph Node Transfer Treatment for Upper Extremity Lymphedema. *J Plast Reconstr Aesthet Surg*. 2022;75(11):3938-3945. doi:10.1016/J.BJPS.2022.08.011
99. Baker A, Kim H, Semple JL, et al. Experimental assessment of pro-lymphangiogenic growth factors in the treatment of post-surgical lymphedema following lymphadenectomy. *Breast Cancer Research*. 2010;12(5):1-17. doi:10.1186/BCR2638/FIGURES/8
100. Kitchen G, Garrett MJ. A trial of intra-lymphatic cyclophosphamide in patients with arm lymphoedema due to metastatic breast carcinoma. *Clin Radiol*. 1971;22(3):379-381. doi:10.1016/S0009-9260(71)80088-0
101. Gousopoulos E, Proulx ST, Bachmann SB, et al. Regulatory T cell transfer ameliorates lymphedema and promotes lymphatic vessel function. *JCI Insight*. 2016;1(16):89081. doi:10.1172/JCI.INSIGHT.89081
102. Liu X, Yuan M, Xiang Q, et al. Single-cell RNA sequencing of subcutaneous adipose tissues identifies therapeutic targets for cancer-associated lymphedema. *Cell Discovery 2022 8:1*. 2022;8(1):1-20. doi:10.1038/s41421-022-00402-5
103. Levi B, Glotzbach JP, Sorkin M, et al. Molecular Analysis and Differentiation Capacity of Adipose-Derived Stem Cells from Lymphedema Tissue. *Plast Reconstr Surg*. 2013;132(3):580. doi:10.1097/PRS.0B013E31829ACE13
104. Ghaleb AM, Yang VW. Krüppel-like factor 4 (KLF4): What we currently know. *Gene*. 2017;611:27. doi:10.1016/J.GENE.2017.02.025

105. Abtahian F, Guerriero A, Sebzda E, et al. Regulation of blood and lymphatic vascular separation by signaling proteins SLP-76 and Syk. *Science*. 2003;299(5604):247-251. doi:10.1126/SCIENCE.1079477
106. Dantas PH dos S, Matos A de O, da Silva Filho E, Silva-Sales M, Sales-Campos H. Triggering receptor expressed on myeloid cells-1 (TREM-1) as a therapeutic target in infectious and noninfectious disease: a critical review. *Int Rev Immunol*. 2020;39(4):188-202. doi:10.1080/08830185.2020.1762597
107. Zhao T, Zhao W, Chen Y, Li VS, Meng W, Sun Y. Platelet-derived growth factor-D promotes fibrogenesis of cardiac fibroblasts. *Am J Physiol Heart Circ Physiol*. 2013;304(12):H1719. doi:10.1152/AJPHEART.00130.2013
108. Mamer SB, Chen S, Weddell JC, et al. Discovery of High-Affinity PDGF-VEGFR Interactions: Redefining RTK Dynamics. *Scientific Reports 2017 7:1*. 2017;7(1):1-14. doi:10.1038/s41598-017-16610-z
109. Brown S, Campbell AC, Kuonqui K, et al. The Future of Lymphedema: Potential Therapeutic Targets for Treatment. *Curr Breast Cancer Rep*. 2023;15(3):1. doi:10.1007/S12609-023-00491-5
110. Jovic D, Liang X, Zeng H, Lin L, Xu F, Luo Y. Single-cell RNA sequencing technologies and applications: A brief overview. *Clin Transl Med*. 2022;12(3). doi:10.1002/CTM2.694
111. Genga RMJ, Kernfeld EM, Parsi KM, Parsons TJ, Ziller MJ, Maehr R. Single-Cell RNA-Sequencing-Based CRISPRi Screening Resolves Molecular Drivers of Early Human Endoderm Development. *Cell Rep*. 2019;27(3):708. doi:10.1016/J.CELREP.2019.03.076