Single-cell analyses of **Ly**mphocytes that infiltrate autoimmunity sites: dissecting immunological mechanisms of **R**heumatoid **A**rthritis - **LYRA**

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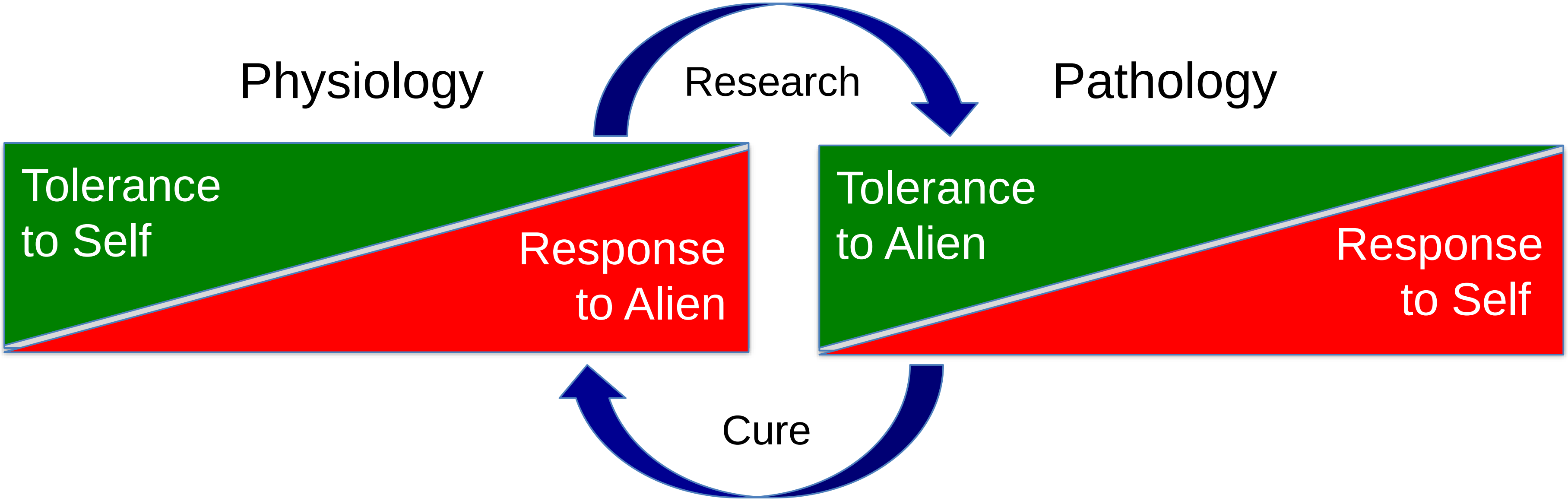
The function of the immune system is altered in cancer (no response) and in autoimmunity (too much response): Why? How to cure it?



- Rheumatoid Arthritis is a devastating disease with a prevalence in the general population around 0.5-1%

- The number of RA patients in Regione Lombardia ranges from 30,000 to 60,000 patients

- RA is among leading causes of chronic disability with a consequent high social and economic cost

Relevant unsolved basic questions and unmet clinical needs of RA

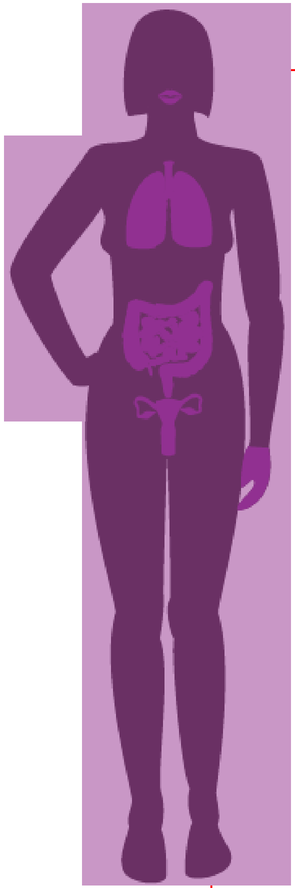
1. Pathobiology: not yet completely understood and implicates local inflammatory response linked to altered intestinal flora;

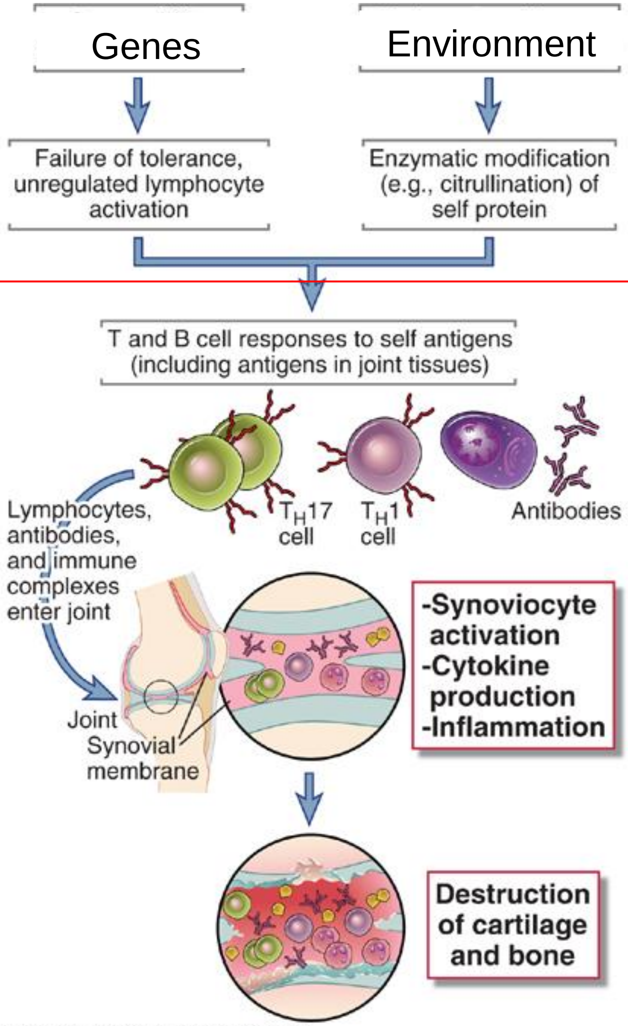
2. Diagnosis, prognosis and prediction of responses to available treatments: lack of specific and sensitive biomarkers;

3. Therapy: the discovery of novel targets may improve medical treatments of RA with higher efficacy and specificity and reduced side effects.

Pathobiology of Rheumatoid Arthritis

Intestinal flora



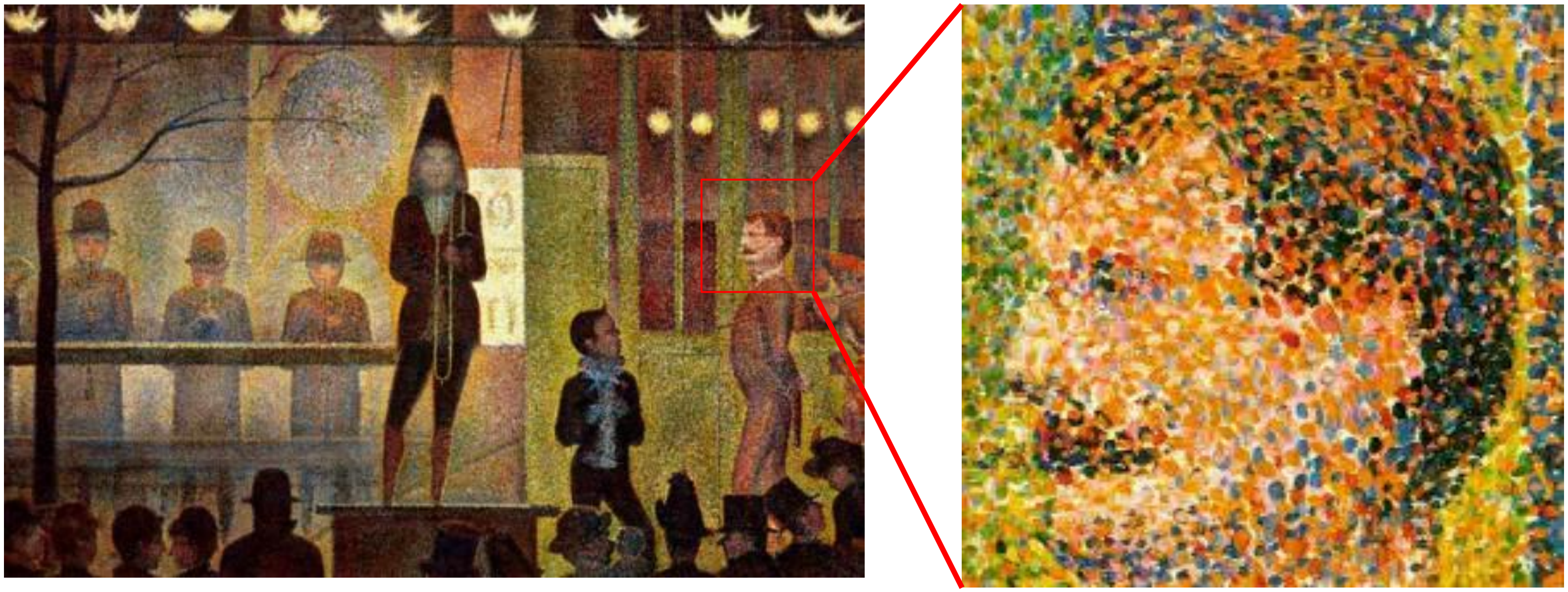


Abbas et al: Cellular and Molecular Immunology, 7e.

Joint (Local)
Inflammation
Immune response
Autoimmunity

Enters Personalized or Precision Medicine

Individuals are made of the sum of different cells

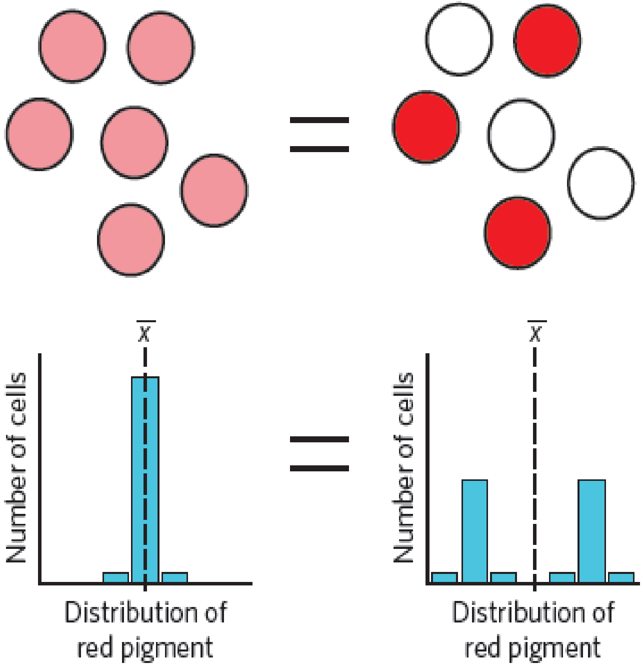


Georges Seurat “La Parade de Cirque” 1888

Single cell analysis to understand the complexity of an organ, function, organism

Why Single Cell analysis?

In a population average, it is not possible to distinguish between a state in which all cells have an intermediate phenotype (pink cells) and one in which few are on (red cells) and other are off (white cells).



This approach is powerful because we can use the genes both to classify cell subpopulations and to interrogate their biological properties

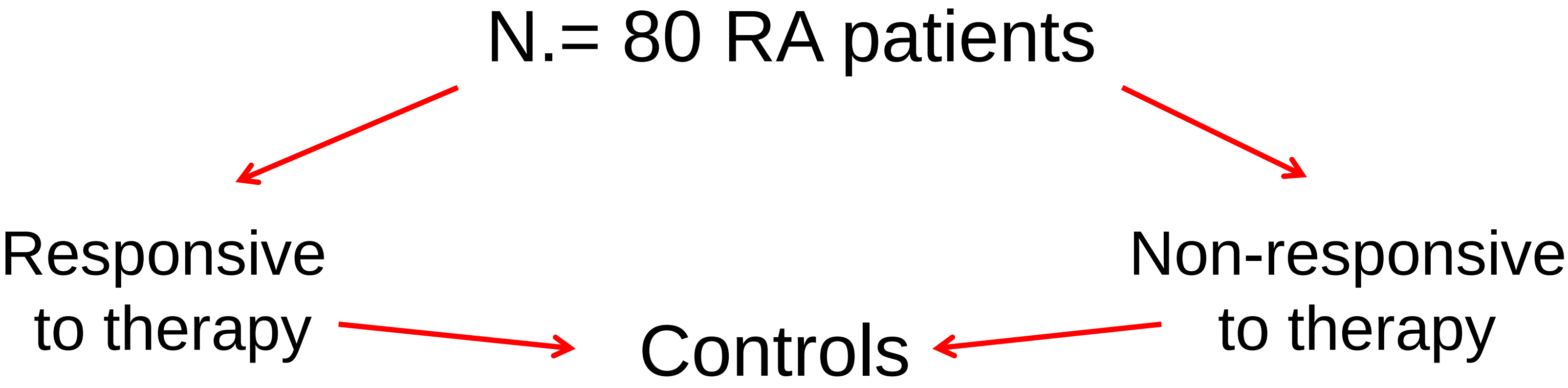
Study design

Developing a new technological platform based on single-cell analysis by

1. Polychromatic flow-cytometry
2. Gene expression
3. Advanced *in vivo* imaging

combined with bulk epigenetic, transcriptional, intestinal microbioma analysis

to determine the role of T lymphocytes in the joints of



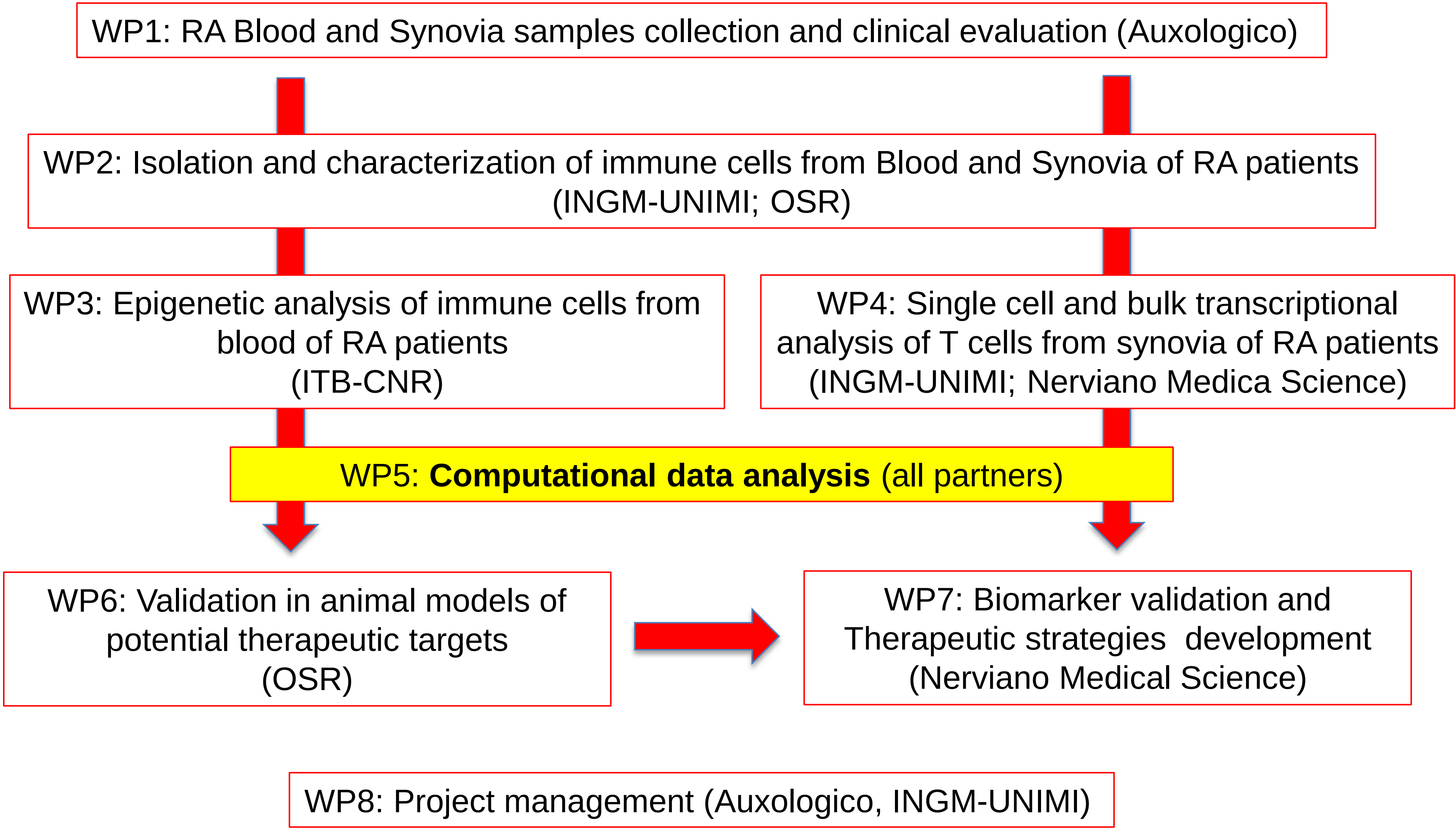
N.= 80 RA patients

Responsive to therapy

Non-responsive to therapy

Controls

Study design



WP1: RA Blood and Synovia samples collection and clinical evaluation (Auxologico)

WP2: Isolation and characterization of immune cells from Blood and Synovia of RA patients (INGM-UNIMI; OSR)

WP3: Epigenetic analysis of immune cells from blood of RA patients (ITB-CNR)

WP4: Single cell and bulk transcriptional analysis of T cells from synovia of RA patients (INGM-UNIMI; Nerviano Medica Science)

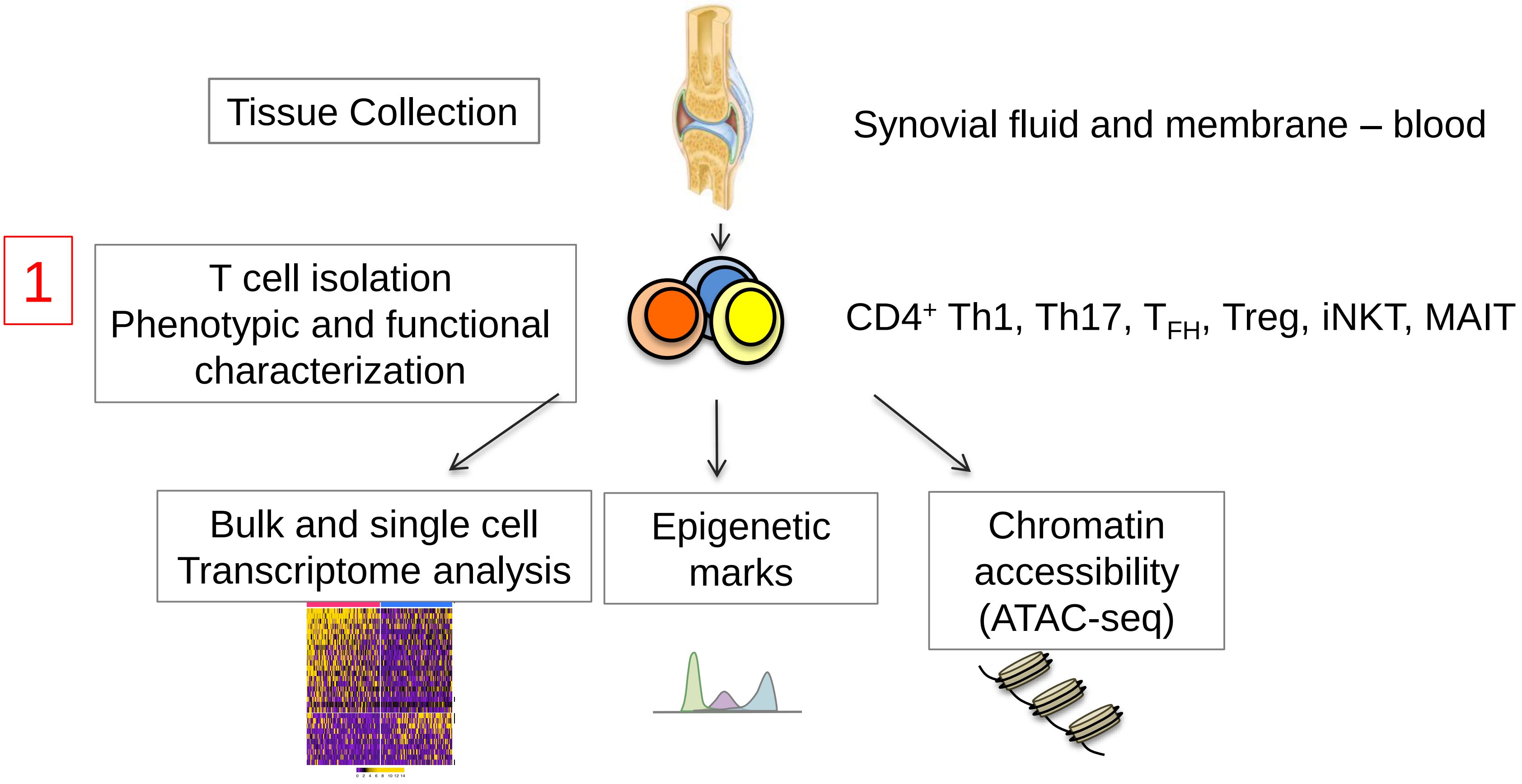
WP5: **Computational data analysis** (all partners)

WP6: Validation in animal models of potential therapeutic targets (OSR)

WP7: Biomarker validation and Therapeutic strategies development (Nerviano Medical Science)

WP8: Project management (Auxologico, INGM-UNIMI)

Molecular and functional characterization of T cell subsets in RA patients



Tissue Collection

Synovial fluid and membrane – blood

1

T cell isolation
Phenotypic and functional characterization

CD4$^+$ Th1, Th17, T$_{FH}$, Treg, iNKT, MAIT

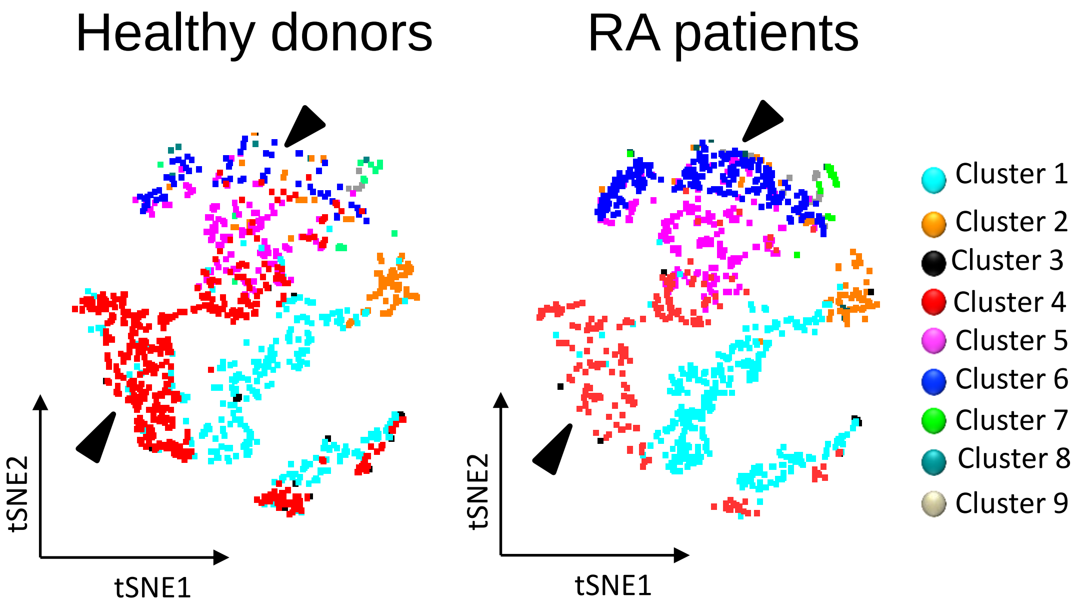
Bulk and single cell Transcriptome analysis

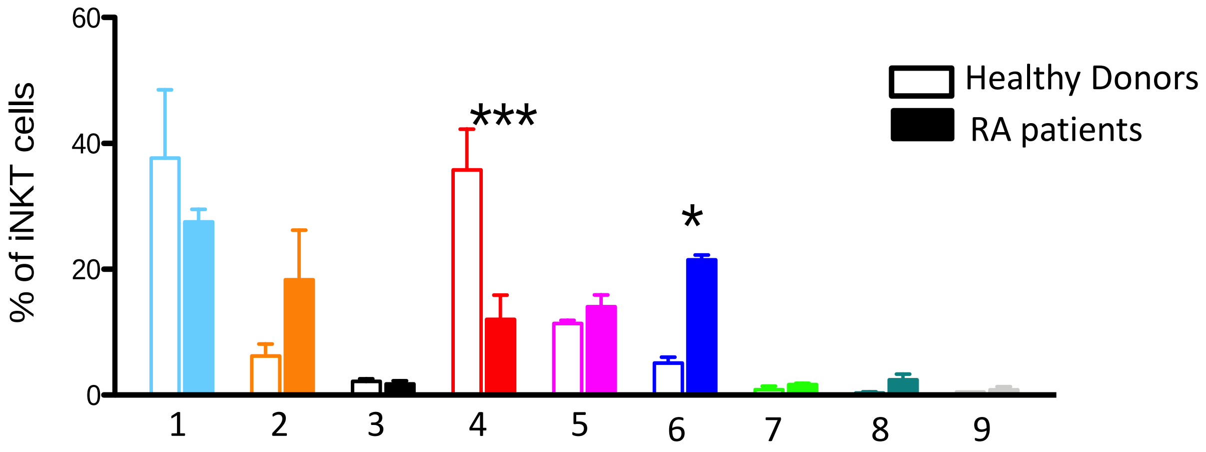
Epigenetic marks

Chromatin accessibility (ATAC-seq)

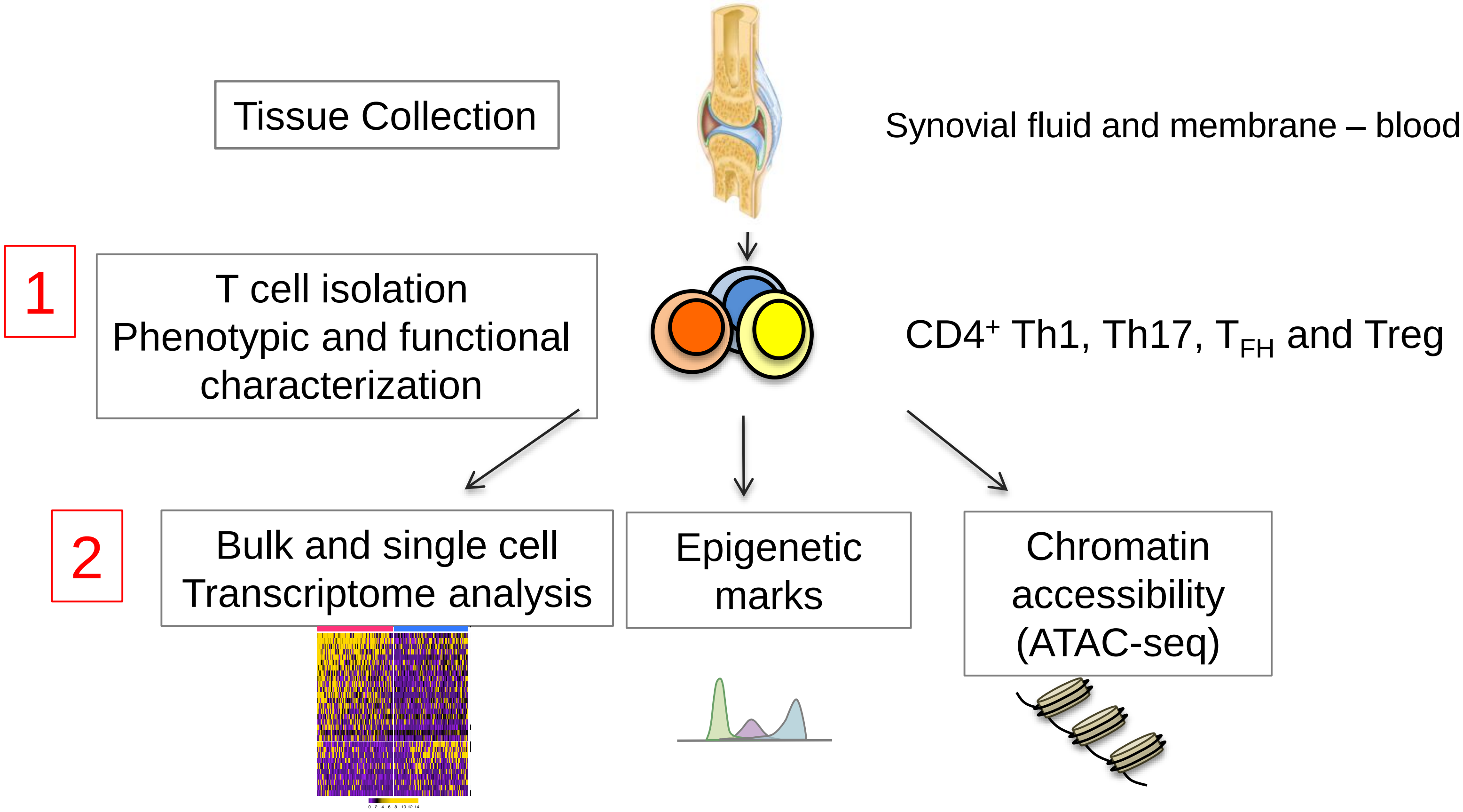
Data integration and reconstruction of the immune landscape in RA patients

1 Example of single cell high dimensional flow cytometry analysis (protein expression,18 markers)





Molecular characterization of CD4$^+$ T cells in RA patients



Tissue Collection

Synovial fluid and membrane – blood

1

T cell isolation
Phenotypic and functional characterization

CD4$^+$ Th1, Th17, T$_{FH}$ and Treg

2

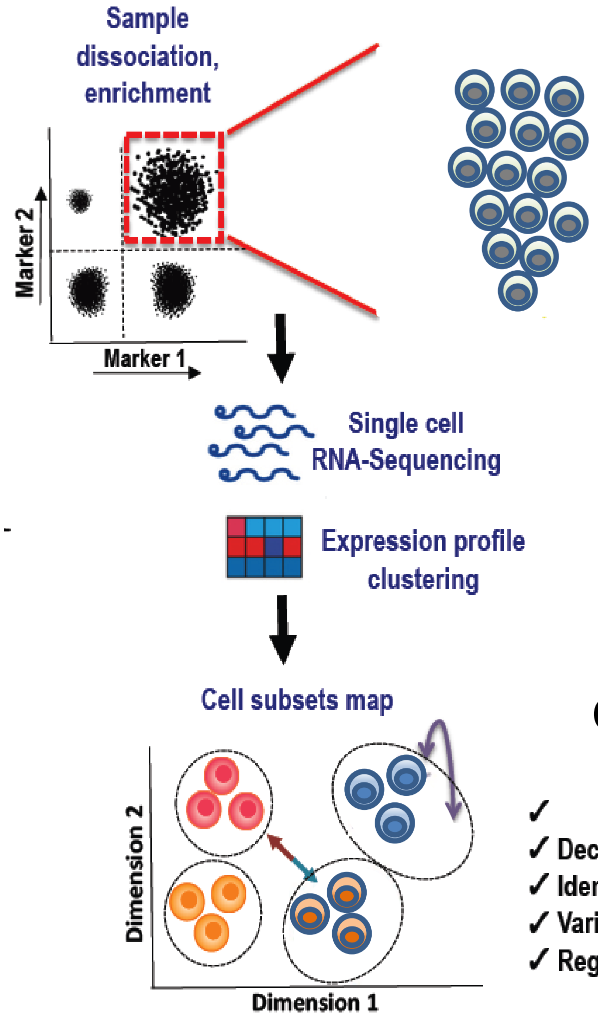
Bulk and single cell Transcriptome analysis

Epigenetic marks

Chromatin accessibility (ATAC-seq)

Data integration and reconstruction of the immune landscape in RA patients

2 Generating the single-cell transcriptional landscape of CD4$^+$ T cells in RA patients

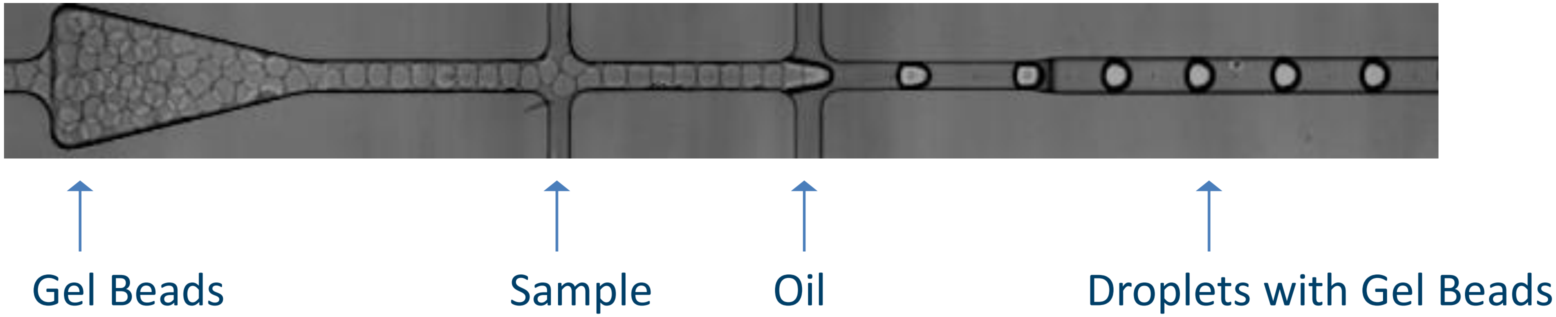


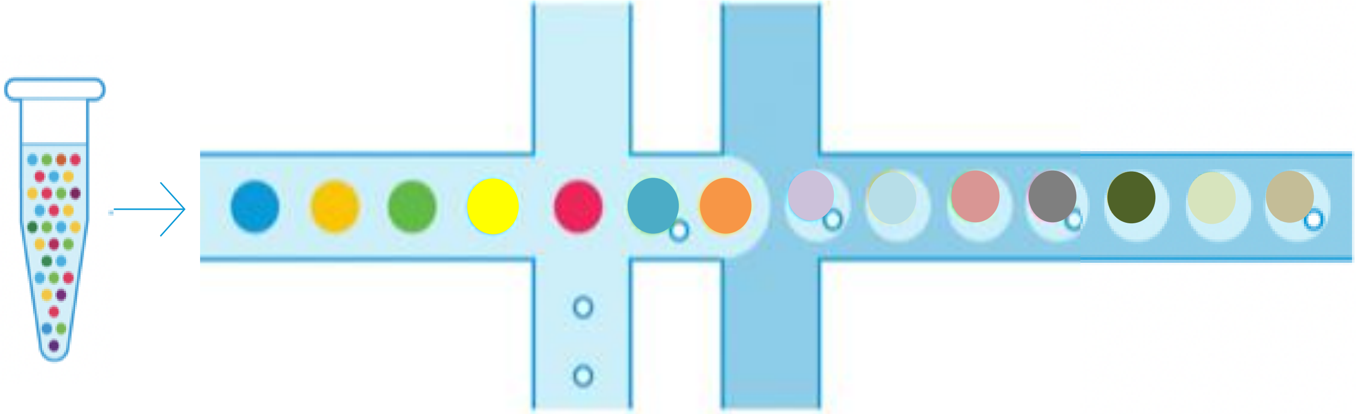
CD4+ T cells form blood or infiltrating synovia of RA patients

CD4$^+$ T cell subsets identification

✓
✓ Deconvolution of population structure
✓ Identification of markers
✓ Variability of transcription
✓ Regulatory network inference

10x Chromium single-cell platform: partitioning with GemCode Technology





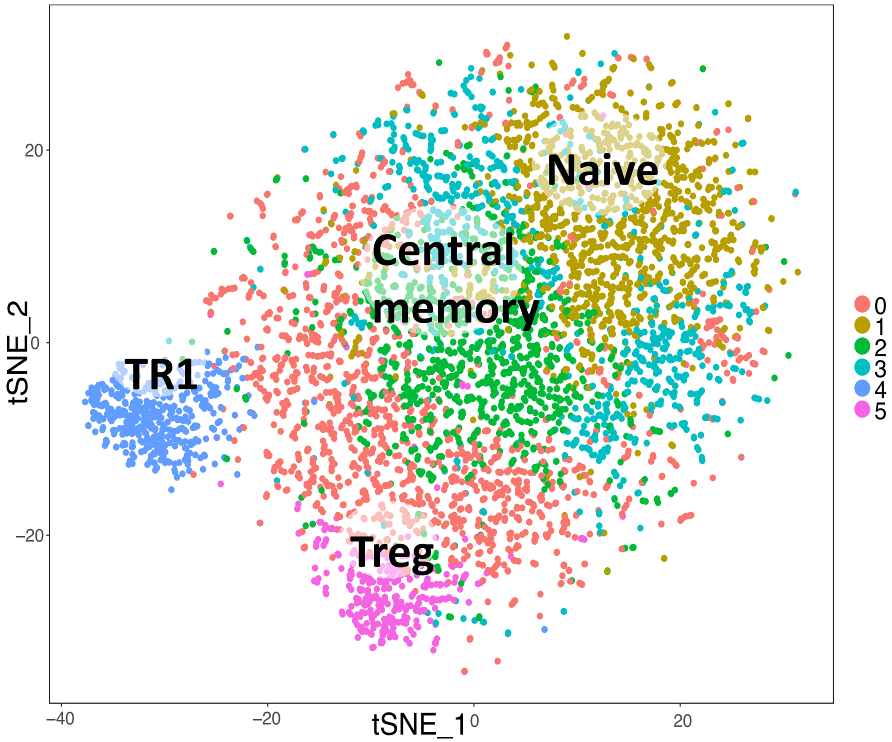
10x Genomics “*Chromium controller*” relies on ad-hoc microfluidic devices that encapsulate single cells in nanoliter droplets along with DNA-barcoded beads. This allows sequencing, in a massively parallel format, transcriptomes from single cells, by pooling thousands of cells into the same sequencing run

Courtesy of 10X Genomics

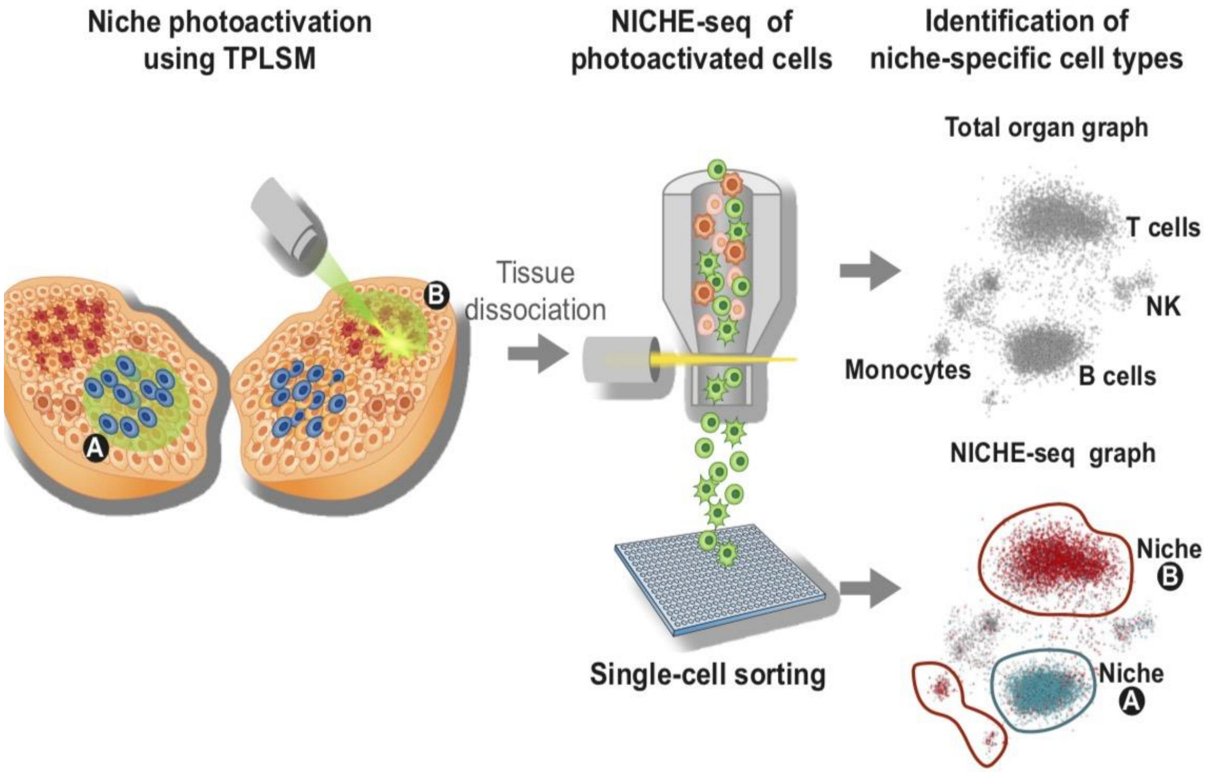
Single CD4$^+$ T cell landscape in blood (gene expression)

KNN graph based clustering on most relevant PCs visualized by t-SNE

Peripheral blood map of healthy donor



3 Example of single cell high dimensional tissue analysis = tissue composition and spatial relationships of single sequenced cells



3 Personalized microbioma (intestinal flora) analysis

4 Translate above findings in diagnostic/prognostic biomarker validation and therapeutic strategies

Possible HTA impact of the results of the project LYRA on RA management

The mean annual social cost of RA was reported € 13,595 per adult patient in Italy

The approximate total economic impact of RA for Regione Lombardia is 700,000,000.00 €/year.

The cost for treating each patient with non-biological drugs is around 400€ and with biologicals 10,000 €/year

Assuming that almost 30% of the RA patients is under biological treatment, the total cost for drugs only is around 160,000,000.00 €/year.

RA patients (Division of Rheumatology, Istituto G. Pini, Milan).
N. 60 established RA patients (disease duration>1 year).

N.20 established RA patients (disease duration>1 year) undergoing arthrocentesis because of an active synovitis with synovial fluid (SF) effusion will be included;
10 patients will be on methotrexate (MTX)
10 on MTX plus a TNF inhibitor treatment
N. 15 patients with an active early RA diagnosed as previously reported with no specific treatment will be investigated (the use of NSAID and corticosteroids (<7.5 mg/day) only will be allowed). Patients with an active synovitis and SF effusion only will be enrolled. Patients will be treated with MTX, antimalarials, Corticosteroids. If non-responsive to MTX after 6 months of follow up a TNF inhibitor will be added to the therapy. The response to therapy will be measured according to American College of Rheumatology / EULAR ACR50 and DAS score27-29. All the RA patients will be regularly followed at the time of the enrollment and after 3, 6 and 12 months of treatment; all the clinical data and adverse/severe adverse events (AE/SAE) will be recorded.
Control groups
: i) 10 active Osteo Arthritis patients undergoing joint surgery;
ii) 10 active Systemic Lupus Erithematosus
; iii) 20 healthy controls.

3

Example of single cell high dimensional tissue analysis = tissue composition

Cell type	tissue	# samples	# captured cells
CD4+ T cells	Synovia (non RA)	1	5000
CD4+ T cells	Synovia RA	3	15000
CD8+ T cells	Synovia RA	1	5000