Identification of pathways represented in liquid biopsy samples from osteoarthritis and autoimmune disease patients by MicroRNA biomarker profiling which could be used for patient stratification in clinical trials

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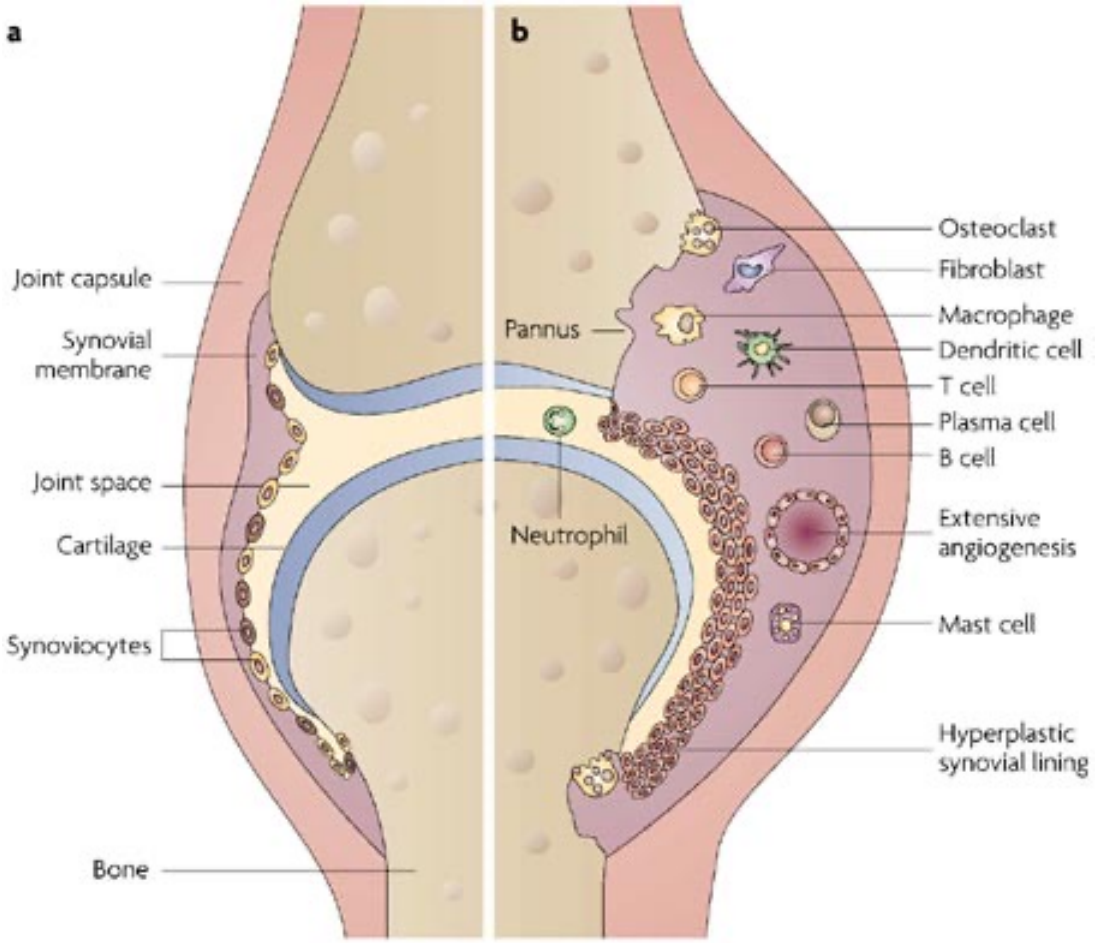
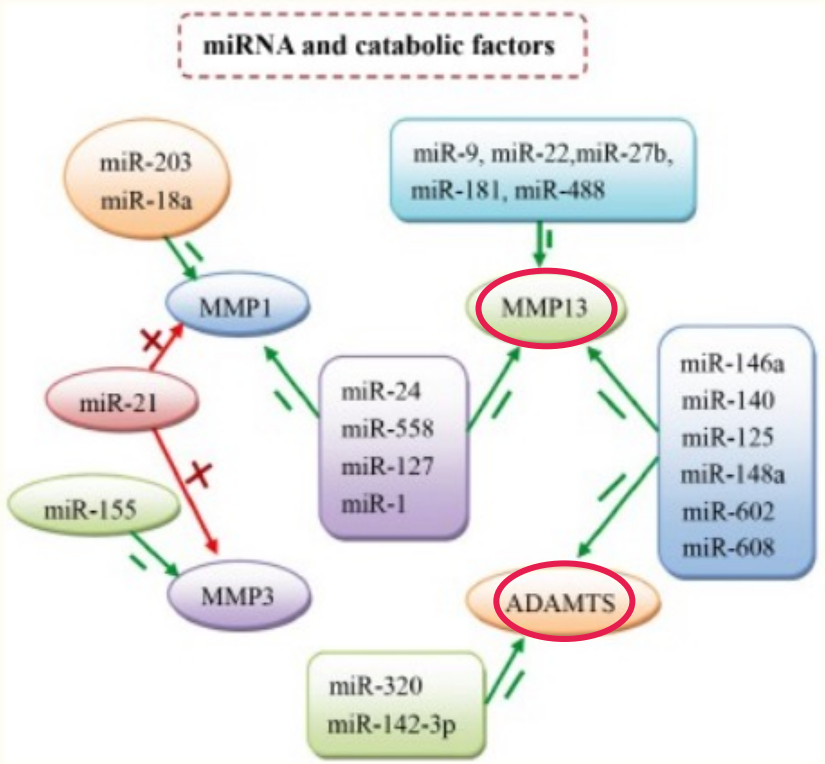
Osteoarthritis (OA) and context of biomarker use



- Osteoarthritis (OA) is the most common form of arthritis, affecting 32.5 million US adults
- Context of biomarker use:
  - Stratification (predictive) biomarkers to focus on special patient subgroup in order to pre-define responders to a specific therapy
- Ideally non-invasively detection of biomarkers in plasma/serum
- Alternatively synovial fluid can be used (synovial fluid contains proteins, miRNAs, exosomes etc. that are released from surrounding tissue (e.g. cartilage, synovium, meniscus)

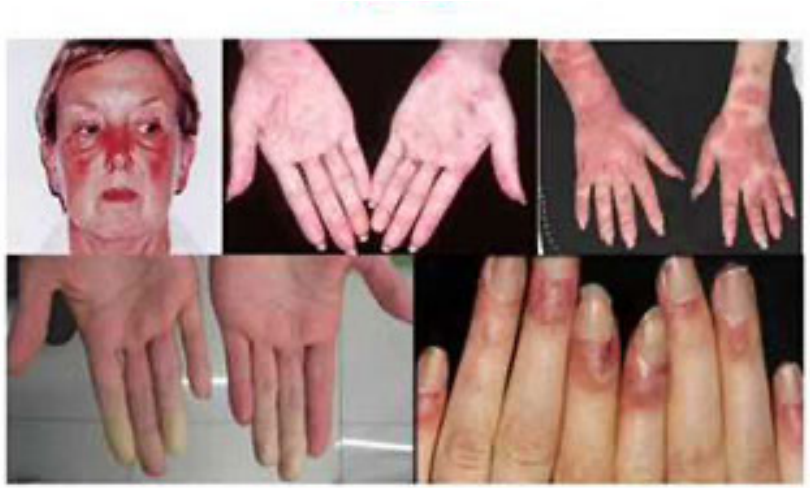
Regulation of proteases involved in OA development by miRNAs



Li et al. 2015

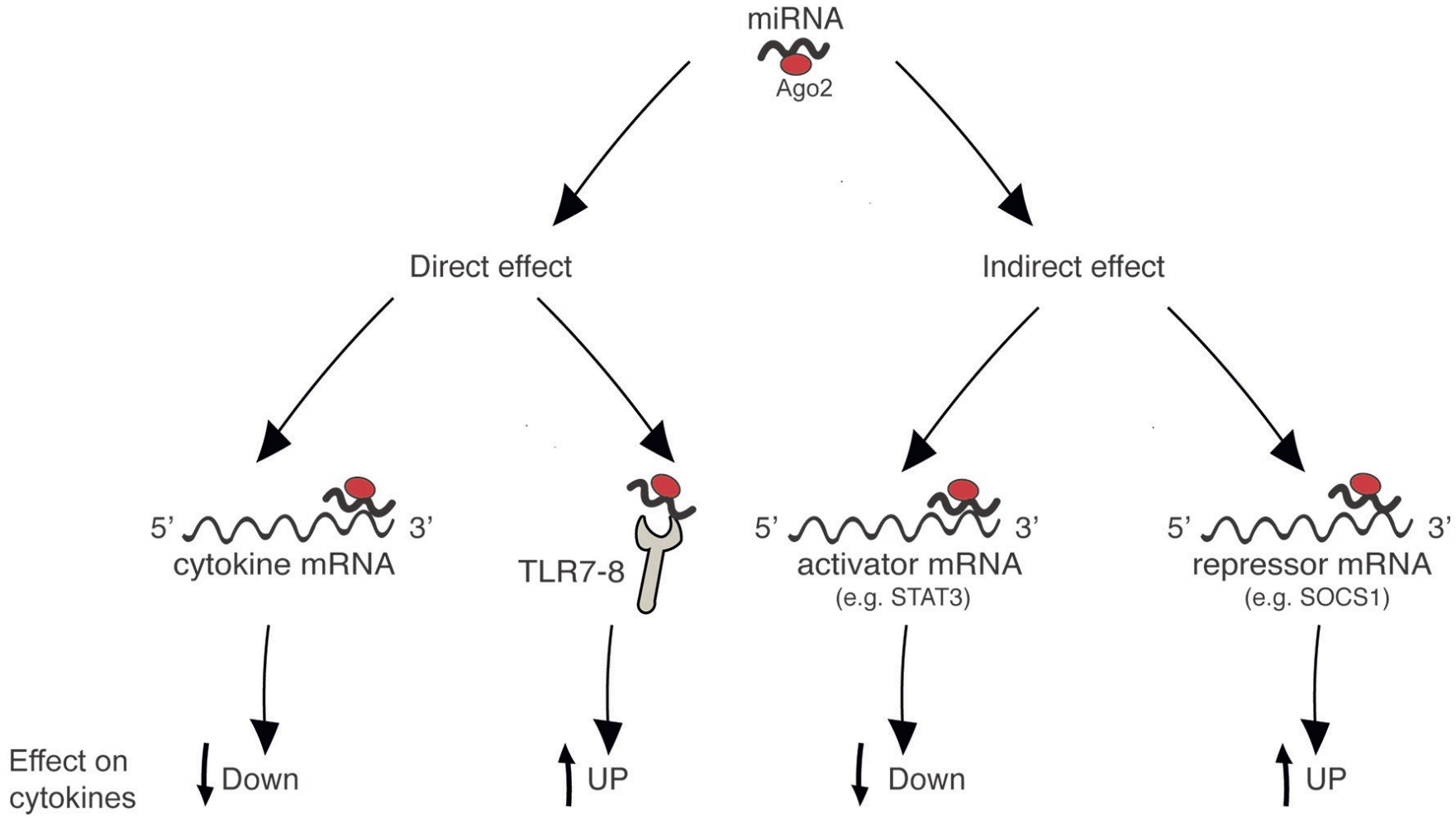
Two of the most prominent proteases involved in pathogenesis of OA (i.e. MMP13 and ADAMTS5) are regulated by microRNAs

Autoimmune disease Systemic Lupus Erythematosus (SLE) and context of biomarker use



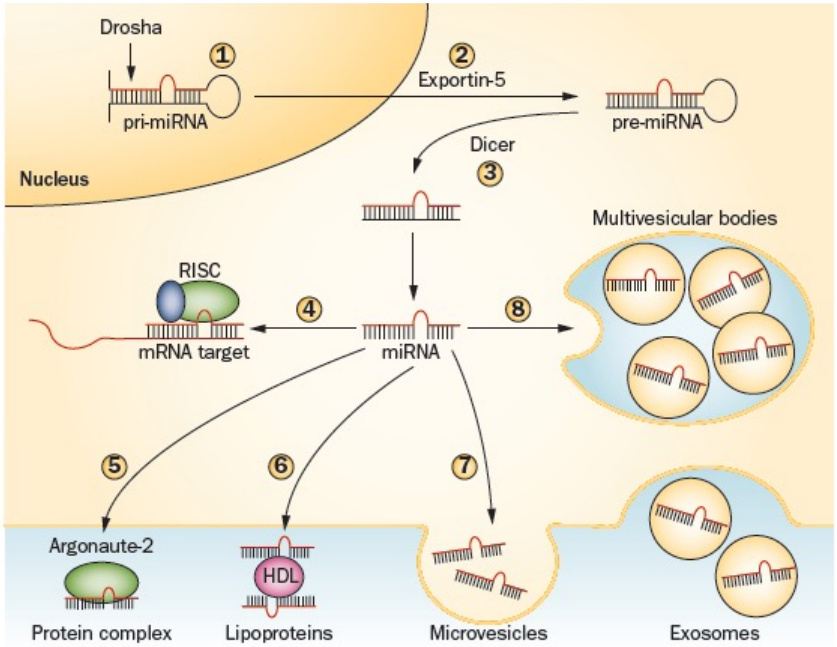
- Systemic lupus erythematosus (SLE) is the most common type of Lupus
- The Lupus Foundation of America estimates that 1.5 million Americans, and at least five million people worldwide, suffer from SLE
- SLE is an autoimmune disease in which the immune system attacks its own tissues, causing widespread inflammation and tissue damage in the affected organs
- It can affect the joints, skin, brain, lungs, kidneys, and blood vessels
- There is no cure for lupus, but medical interventions and lifestyle changes can help control it
- Context of biomarker use:
  - Stratification (predictive) biomarkers to focus on special patient subgroups in order to pre-define responders to a specific therapy in clinical trials
- Ideally non-invasively detection of biomarkers in plasma/serum (liquid biopsy)

Mechanism of cytokine regulation by miRNAs (e.g. in autoimmune diseases)



https://www.frontiersin.org/articles/10.3389/fimmu.2019.00015/full

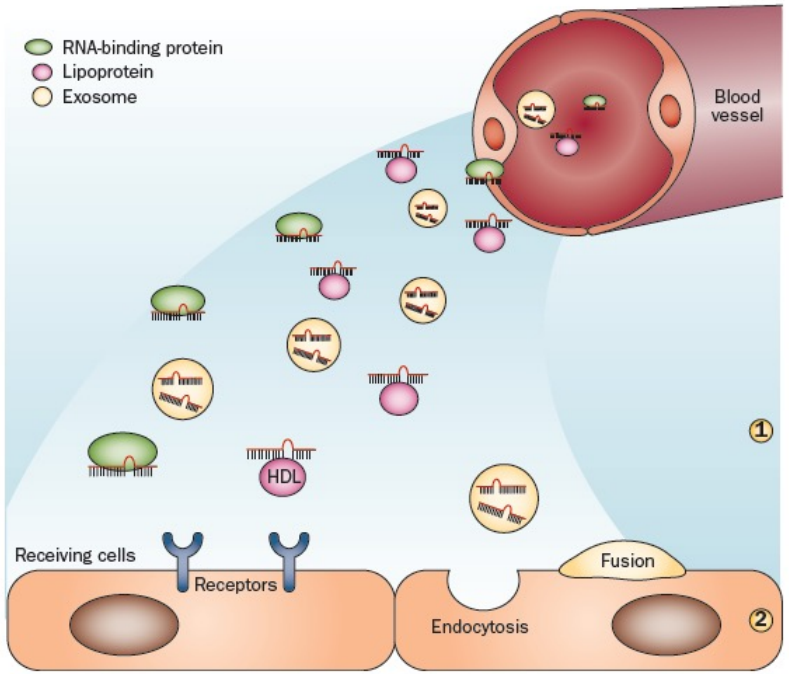
Biogenesis and extracellular release of microRNAs (miRNAs)



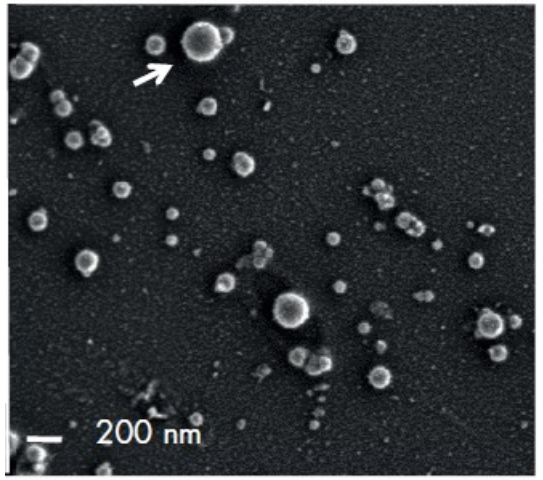
From: Guay and Regazzi, Nature Reviews 2013

- MicroRNAs are transcribed from intergenic or intronic sequences of coding genes as long precursor transcripts
- Mature ~20 nt long ss miRNAs act by guiding the RISC complex to complementary sequences within the 3´untranslated region of target mRNAs leading to translational repression of target transcripts
- Some miRNAs are tissue specific (e.g. miR-122 in liver)
- A single miRNA can potentially bind and regulate the expression of hundreds of cellular mRNA targets and associated pathways
- Alternatively, mature miRNAs can be loaded into microvesicles, exosomes or protein complexes and released from cells as circulating miRNAs into the blood stream

Vesicle-bound circulating microRNAs (miRNAs) are stable and detectable in liquid biopsy



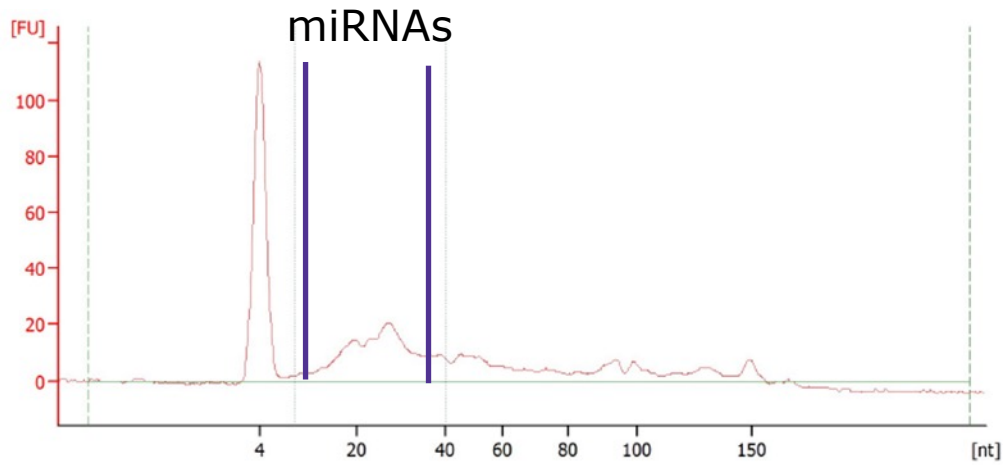
Exosomes (microvesicles of 100-200nm diameter)

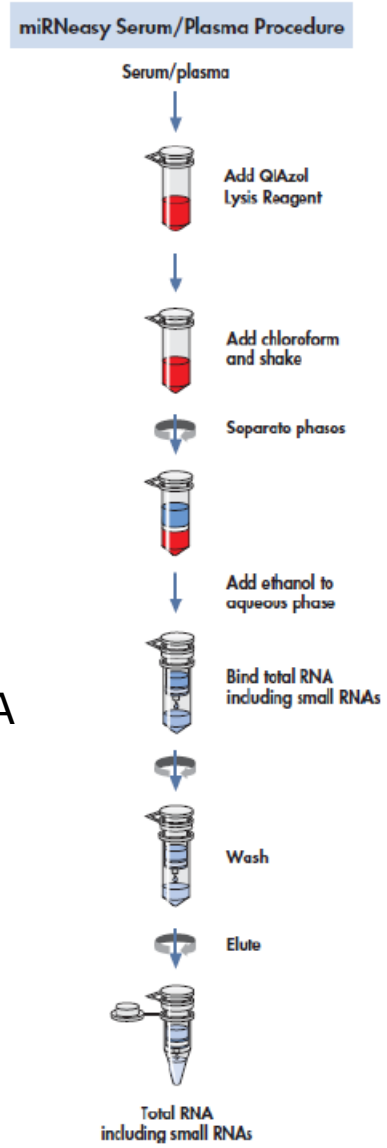


From: Guay and Regazzi, Nature Reviews 2013

- Circulating miRNAs that are bound to proteins and microvesicles/exosomes are efficiently protected from nuclease degradation e.g. in blood
- After extraction from liquid biopsy, miRNAs can be stored stable at -80°C and quantified by transcriptional assays (e.g. qPCR, NGS) with high sensitivity

Extraction of microRNAs from liquid biopsy using miRNeasy kit (Qiagen) and QC

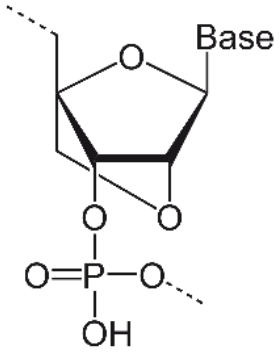




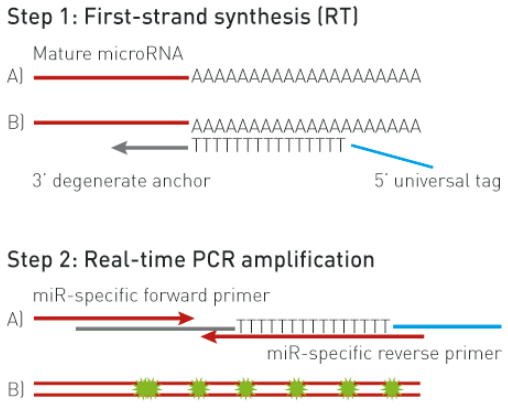
- „Small RNA" Bioanalyzer assay (Agilent) can be used for QC of miRNA preparations
- Alternatively, spike-in oligos can be added to liquid biopsy and RNA extraction efficiency can be checked by qPCR analysis
- Usually 200µl of liquid biopsy are sufficient for microRNA preparation

Expression analysis of microRNAs using LNA oligos and qPCR assay (Qiagen)

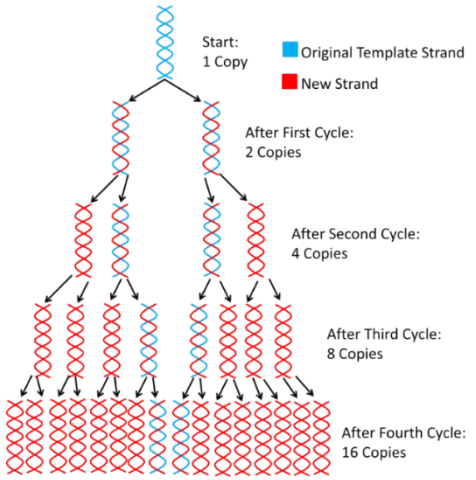
miRNome LNA™ (Exiqon/Qiagen)



LNA (Locked Nucleic Acid)

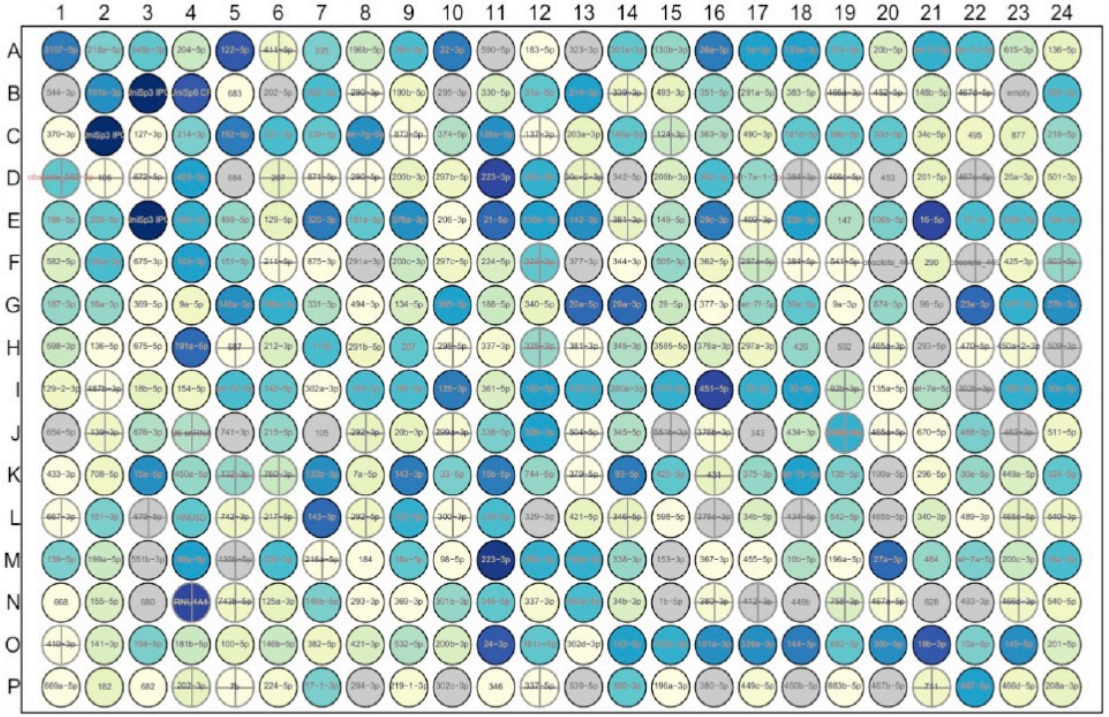


PCR amplification of microRNA templates



- LNA oligonucleotides have a special („locked") conformation which allows them to bind short RNA molecules (i.e. miRNAs) with high affinity
- This results in higher sensitivity and specificity of microRNA expression profiles compared to other technologies (Mestdagh, P. et al. (2014) Evaluation of quantitative miRNA expression platforms in the microRNA quality control (miRQC) study, *Nature Methods* **11** (8), 809.)
- No need for pre-amplification steps

Expression analysis of microRNAs using LNA oligos and qPCR



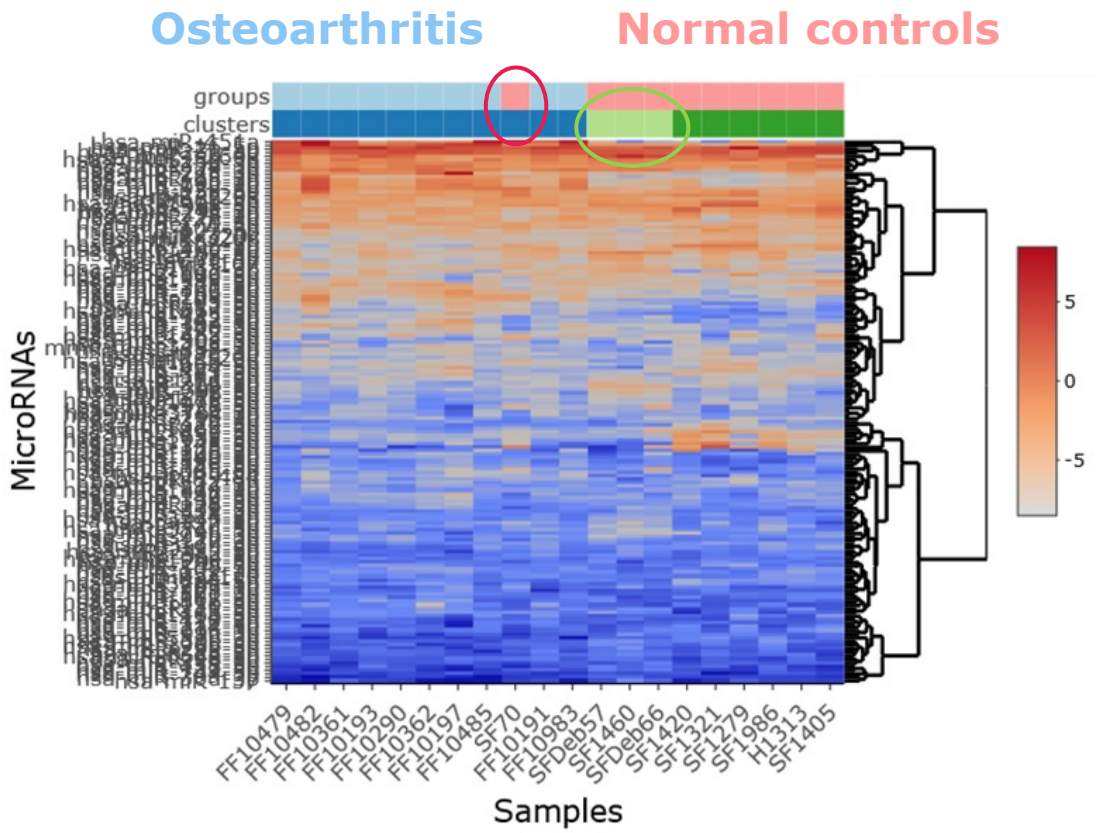
- 752 human miRNAs were tested on miRNome LNA miRNA PCR panel I + II (384-well format, one data point per microRNA)
- RNA isolation, cDNA synthesis and qPCR steps were monitored using QC spike-ins
- Global expression data normalization was performed based on the average of miRNA assays detected in all samples

MicroRNA studies performed using Qiagen LNA technology

- MicroRNA expression profiling of human synovial fluid (SF) samples (Osteoarthritis):
  - Comparison between normal (post mortem) synovial fluid vs. osteoarthritis samples (N=10/group)

- Profiling of circulating microRNAs in human plasma samples (Systemic Lupus Erythematosus (SLE) and Lupus Nephritis (LN)):
  - Plasma samples from 46 subjects (MGH collaboration):
    - Healthy controls (N=18)
    - Lupus Nephritis patients (N=8, various SLEDAI scores)
    - SLE patients (N=20, various SLEDAI scores)

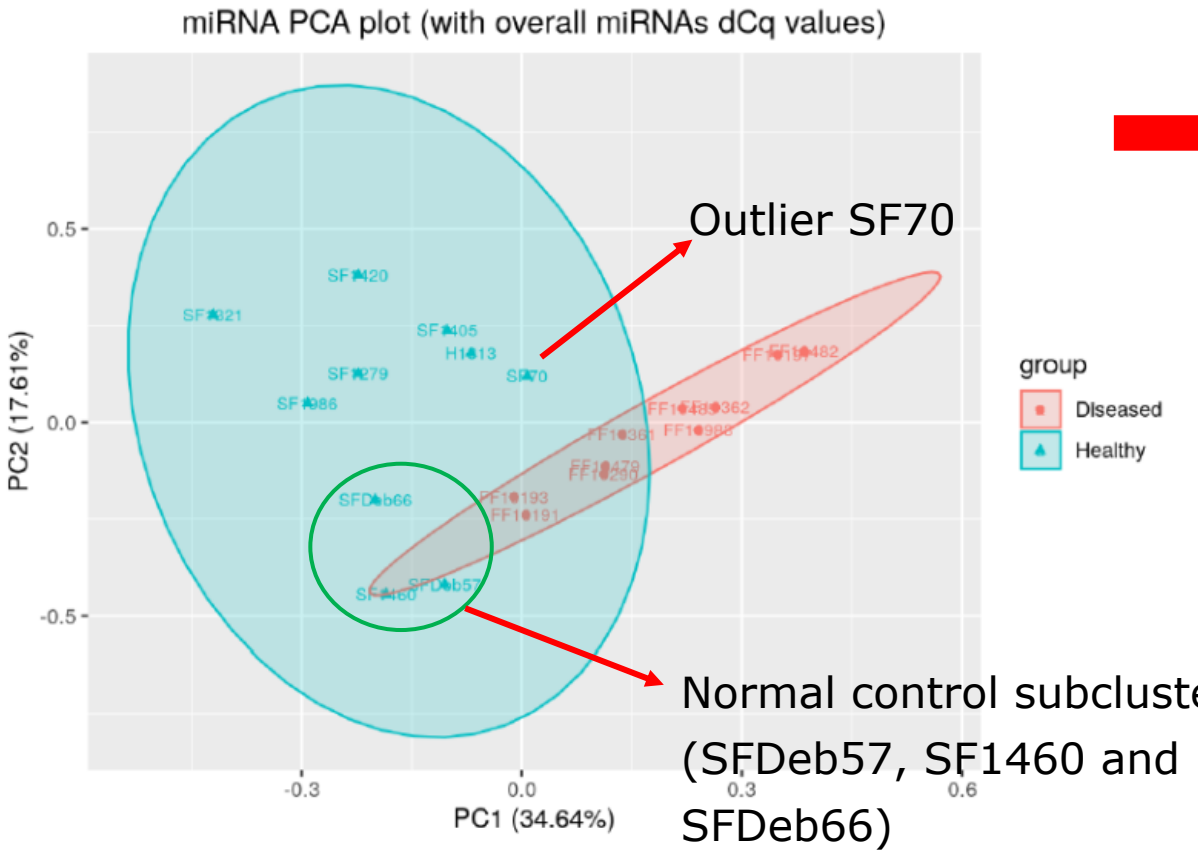
Osteoarthritis: 2D-Hierarchical clustering analysis of microRNA expression profiles in SF samples



(red colour represents expression levels above global mean, blue below)

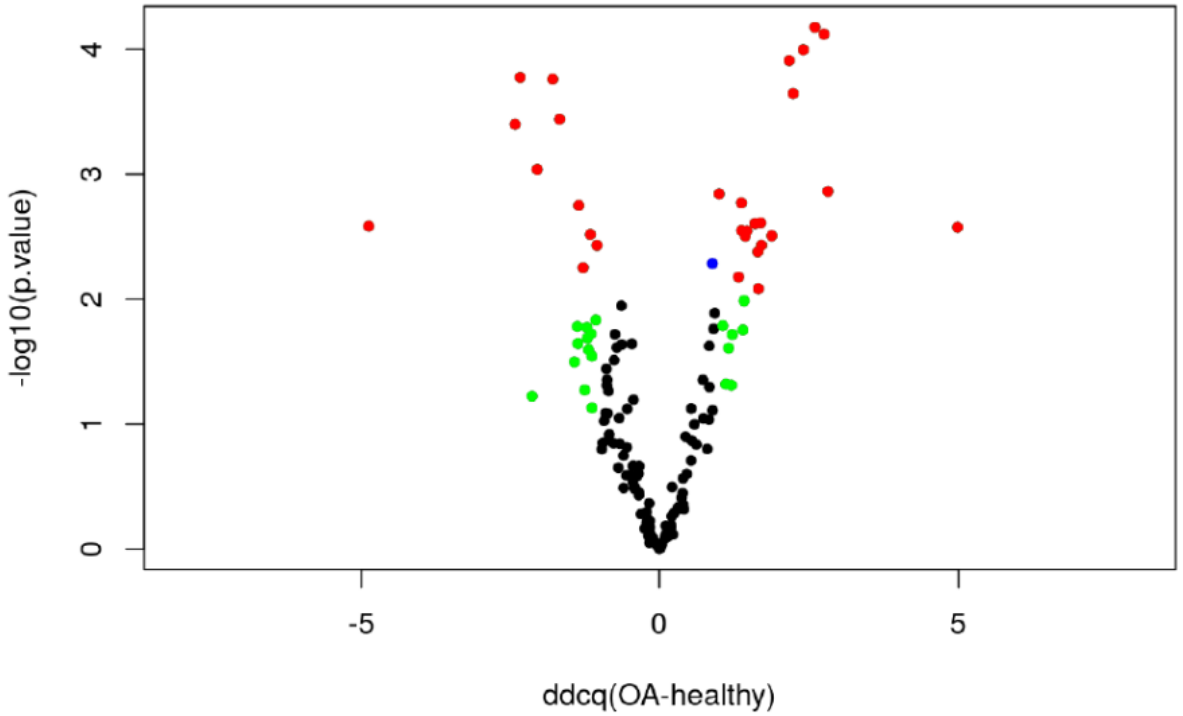
- MicroRNA expression profiles showed a clear separation between biological groups (OA vs. normal controls)
- Sample SF70 from normal controls seemed to be an outlier (red circle)
- Samples SFDeb57, SF1460 and SFDeb66 (green circle) formed a subcluster in normal control group (could be induced by metabolic syndrome or inflammation processes in knee joint)

Osteoarthritis: PCA clustering analysis of microRNA expression profiles in SF samples



- As observed in 2D-hierarchical clustering analysis, miRNA expression profiles of all 10 OA samples formed a compact cluster (red circle)
- In contrast, the normal control group formed a more heterogenous cluster (blue circle) in which again miRNA expression profiles of "controls" SFDeb57, SF1460 and SFDeb66 seemed to be more similar to OA samples than the remaining normal control samples

Osteoarthritis: Vulcano plot of differentially expressed microRNAs in SF samples



Red dots: Highly significant regulated microRNAs in SF samples (10 OA vs. normal subjects):

- T-test p-value <0.01
- Benjamini-Hochberg FDR <0.05
- Absolute fold-change >1.8
- Detectable in at least 18 out of 20 samples

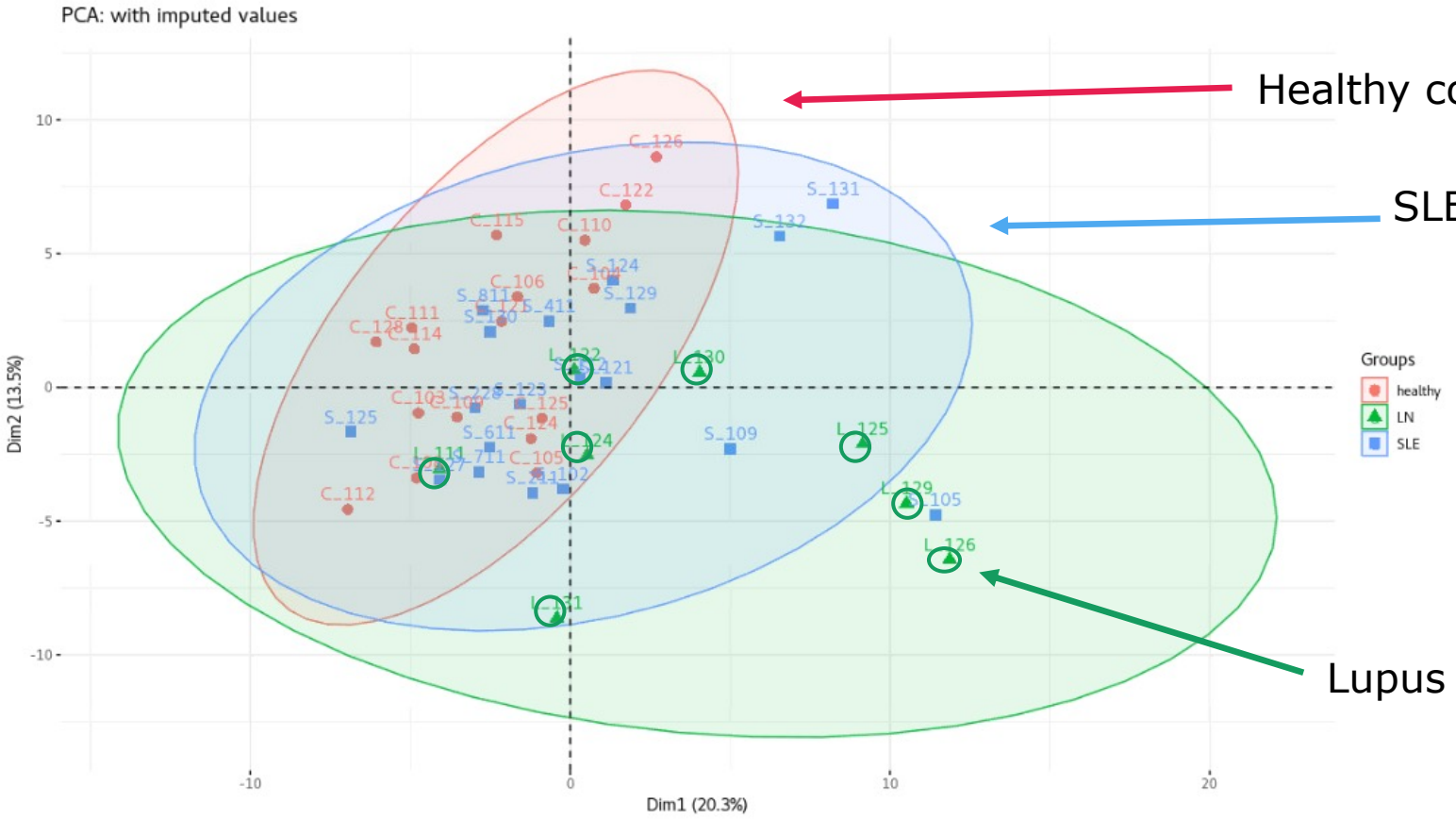
14 out of the top 29 regulated miRNAs were already described in literature in the OA/chondrocyte context

Osteoarthritis: Identification of OA relevant pathways regulated by microRNAs differentially expressed in SF samples

Pathway	Total # of miRNAs involved in pathway	P-value	FDR
SIGNALING_BY_BMP	45	0	0
TGF_BETA_RECEPTOR_SIGNALING_ACTIVATES_SMADS	49	0	0
ACTIVATED_TLR4_SIGNALING	87	0	0
APOPTOSIS	98	0	0
IL1_SIGNALING	52	0	0
SIGNALING_BY_FGFR	92	0	0
CYTOKINE_SIGNALING_IN_IMMUNE_SYSTEM	101	0.001	0.008

- Affected pathways were identified by „MicroRNA Set Enrichment Analysis" (MiRSEA)
- BMP, TGFb, Apoptosis, FGF and cytokine signaling pathways are highly relevant for OA development
- Pathway related miRNAs were highly significant (FDR< 0.05) regulated in all OA patient samples which were also well separated from controls in clustering/PCA analysis
- Consequently these subjects should respond to treatment which modifies identified pathways (e.g. BMP, TGFb and FGFR signaling)

Autoimmune disease: MicroRNA profiling of plasma samples from SLE and Lupus Nephritis patients vs. healthy controls



Healthy controls

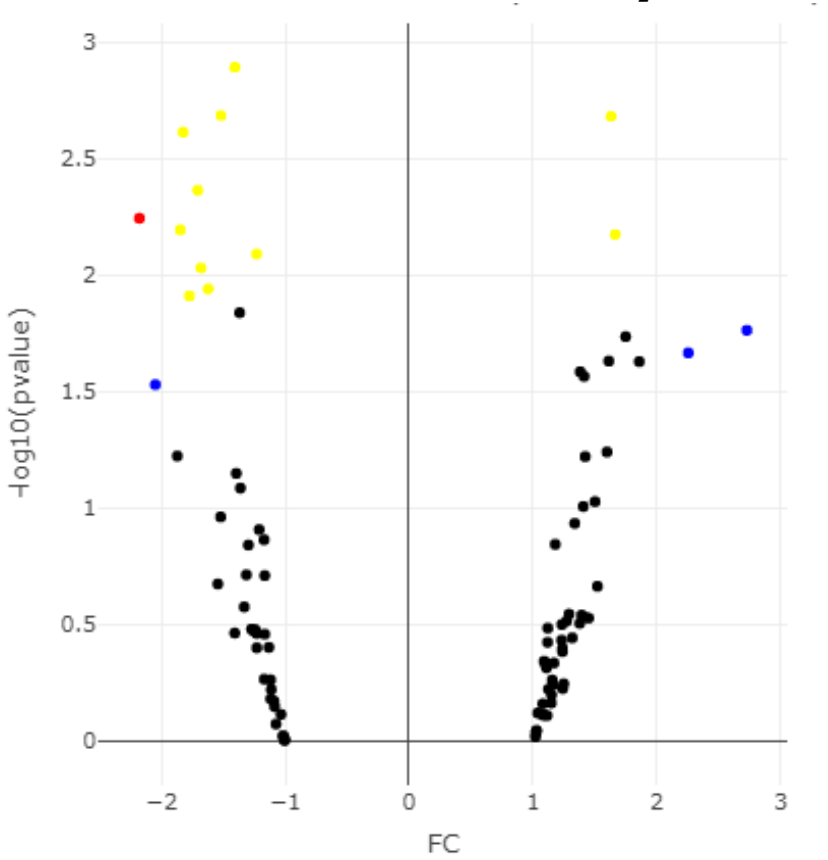
SLE patients

Lupus Nephritis patients

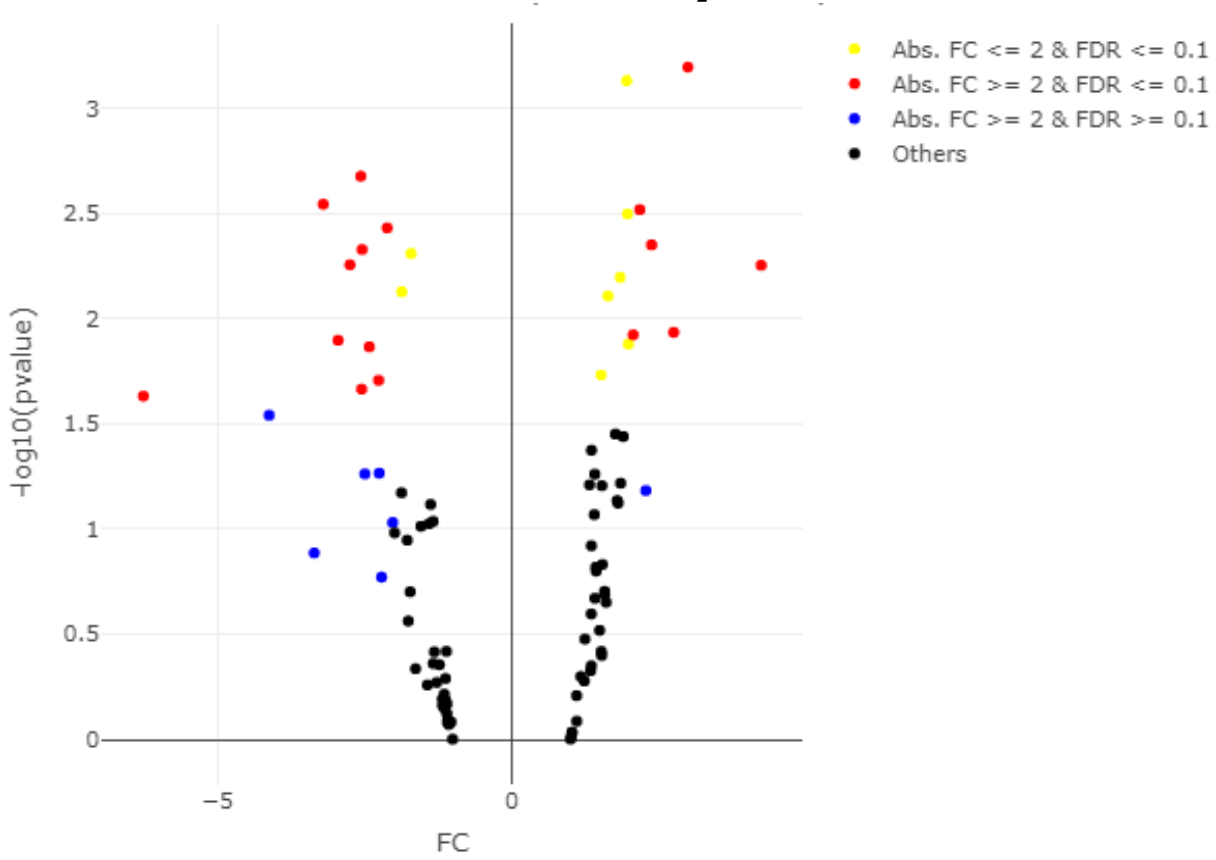
- In PCA analysis miRNA profiles of healthy controls (red circle) and SLE patients (blue circle) do overlap
- Lupus Nephritis patient cluster (green circle) is partially separated from healthy controls indicating disease-related differential miRNA expression

Autoimmune disease: Identification of differentially expressed miRNAs

SLE vs. Healthy



LN vs. Healthy



- SLE vs. Healthy: 12 microRNAs with False Detection Rate (FDR)< 0.1
- LN vs. Healthy: 24 microRNAs with FDR< 0.1
- 10 miRNAs were overlapping

Autoimmune disease: Pathway Analysis (Lupus Nephritis vs. Controls)

Pathways	Total # of miRNAs involved in pathway	P-value	FDR
KEGG_ANTIGEN_PROCESSING_AND_PRESENTATION	44	0	0
KEGG_ARGININE_AND_PROLINE_METABOLISM	27	0	0
KEGG_INTESTINAL_IMMUNE_NETWORK_FOR_IGA_PRODUCTION	16	0	0
KEGG_TYROSINE_METABOLISM	12	0	0
KEGG_SYSTEMIC_LUPUS_ERYTHEMATOSUS	42	0.001	0.017
KEGG_HEDGEHOG_SIGNALING_PATHWAY	27	0.003	0.036
KEGG_GLYOXYLATE_AND_DICARBOXYLATE_METABOLISM	12	0.003	0.036
KEGG_AMINOACYL_TRNA_BIOSYNTHESIS	29	0.004	0.042
KEGG_TOLL_LIKE_RECEPTOR_SIGNALING_PATHWAY	59	0.005	0.047
KEGG_ALANINE_ASPARTATE_AND_GLUTAMATE_METABOLISM	24	0.006	0.050
KEGG_CHEMOKINE_SIGNALING_PATHWAY	69	0.007	0.053

- IgA production, TLR and chemokine signaling pathways are highly relevant for SLE/LN development
- Antigen processing and presentation pathway could be linked to production of auto-antibodies in SLE/LN
- Pathway related miRNAs were highly significant (FDR< 0.1) regulated in 5 LN patient samples that showed highest separation from control group in PCA plot
- Consequently these subjects should respond to treatment which modifies identified pathways (e.g. cytokine and TLR signaling)

Autoimmune disease: Pathway Analysis (SLE vs. Controls)

Pathways	Total # of miRNAs involved in pathway	P-value	FDR
KEGG_TYROSINE_METABOLISM	12	0	0
KEGG_DRUG_METABOLISM_CYTOCHROME_P450	17	0.001	0.042
KEGG_ARGININE_AND_PROLINE_METABOLISM	27	0.002	0.050
KEGG_INTESTINAL_IMMUNE_NETWORK_FOR_IGA_PRODUCTION	16	0.003	0.050
KEGG_RETINOL_METABOLISM	16	0.003	0.050
KEGG_HEDGEHOG_SIGNALING_PATHWAY	27	0.004	0.053
KEGG_ALANINE_ASPARTATE_AND_GLUTAMATE_METABOLISM	24	0.005	0.053
KEGG_PORPHYRIN_AND_CHLOROPHYLL_METABOLISM	21	0.005	0.053
KEGG_ANTIGEN_PROCESSING_AND_PRESENTATION	44	0.006	0.056
KEGG_FC_EPSILON_RI_SIGNALING_PATHWAY	46	0.007	0.059

- IgA production pathway is highly associated with nephropathy in SLE/LN
- Antigen processing and presentation pathway could be linked to production of auto-antibodies in SLE/LN
- As LN is a more progressive and severe form of Lupus, presumably regulated microRNAs affecting TLR and cytokine signaling pathways could not be identified in SLE patients

Summary I

- Expression profiling of microRNAs using LNA qPCR technology in liquid biopsy samples:
  - Synovial fluid from osteoarthritis patients and normal controls (N=10 each)
  - Plasma samples from SLE patients (N=20), LN patients (N=8) and healthy controls (N=18)
- 2D-hierarchical and PCA clustering of microRNA expression profiles showed that all OA samples formed a compact cluster. However, clustering of control samples revealed subclusters which could indicate very early stages of OA already induced in „normal" samples by e.g. metabolic syndrome or inflammation processes
- In PCA analysis of miRNA profiles from subjects with autoimmune disease, expression profiles of SLE patients did overlap with healthy controls. Profiles of Lupus Nephritis patients partially separated from healthy controls indicating disease-related differential miRNA expression
- Pathway analysis demonstrated that pathways highly relevant for the development of OA (e.g. BMP-, TGFbeta- and FGFR-signaling) and SLE/LN (e.g. antigen processing and presentation, TLR and cytokine signaling) could be dysregulated by microRNAs identified in this study
- For osteoarthritis and SLE/LN indication, microRNAs which are involved and affecting disease-relevant pathways were highly significant regulated in subjects that were most separated from control groups in PCA plots

Summary II

In the context of biomarker use microRNA expression profiles derived from liquid biopsy samples could be used for:

- Identification of disease-relevant pathways and novel targets involved in these pathways
- Stratification of patients in clinical trials after treatment with compounds which interfere with disease-relevant pathways:
  - Selection of subjects where pathway associated miRNAs are regulated with high significance (FDR< 0.1) and are significantly separated from control group in PCA/clustering analysis of miRNA expression profiles
  - Patients selected based on this analysis should respond better to pathway modifying drug treatment:
    - Osteoarthritis samples: Results from this study suggest that OA patients might well respond to drugs interfering with BMP, cytokine and FGFR signaling
    - SLE/LN samples: Results suggest that LN patients might respond better to drugs interfering with cytokine and TLR signaling compared to SLE patients

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