Catching the Th17 transformers: acting on the cytokine panel that drives Th17 plasticity, a winning combination for IFN-γ mediated (skin) disorders.

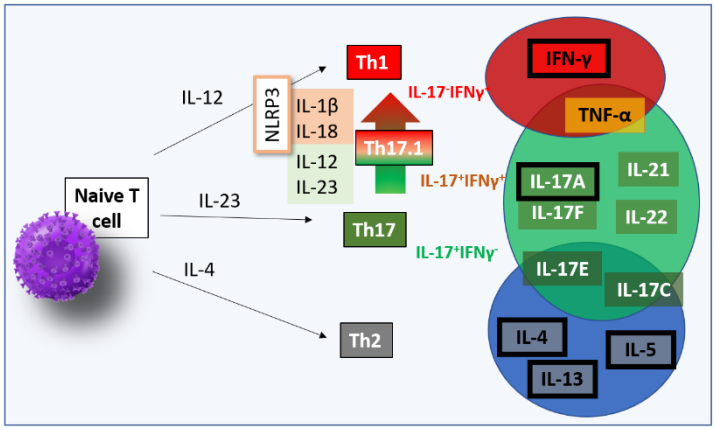
Rationale and positioning with regard to state-of-the-art: PROJECT OUTLINE

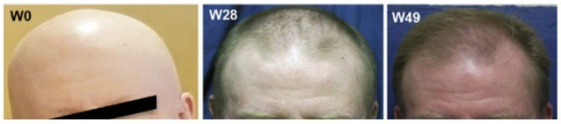
Vitiligo and alopecia areata share pathogenic similarities and both disorders can occur simultaneously. Toxic epidermal necrolysis is an often lethal IFN-γ dominant skin disorder, resulting from a type IV hypersensitivity response against drugs. To date, supportive therapy is the standard of care.

Example of vitiligo, alopecia areata and toxic epidermal necrolysis illustrating the auto-immune destruction of melanocytes, hair follicles and keratinocytes, respectively,

In this project, we will study if a combination of inhibiting the cytokine panel that drives Th17 plasticity is an effective and feasible strategy to dampen the auto-immune based destruction of epidermal cells.

Increasing evidence documents that shifts in Th1/T2/Th17 ratios, as observed in several autoimmune disorders, are largely dependent on the plasticity of the Th17 pathway rather than from the differentiation of naïve T cells into effector T cells. Th17 lymphocytes retain stem cell-like properties which allows them to acquire a Th1-like profile illustrated by the production of IFN-γ while losing their capacity to release IL-17A. This fast mechanism is necessary to react quickly to pathogens as differentiation of naïve T cells to effector T helper cells takes time (around 7-14 days). The key cytokines that drive the transformation of Th17 cells into non-conventional Th1-cells are IL-1β, IL-12, IL-18 and IL-23 in a low TGF-β immune environment. To date a treatment approach that targets all key cytokines involved in Th17>Th1 plasticity has not been attempted. Inhibiting IL-12/IL-23 alone has lead to variable results. NLRP3 signaling is elevated in vitiligo and alopecia areata which is important for the production of IL-1β and IL-18. From a genetic point of view, polymorphisms enhancing AHR expression are protective for vitiligo and AHR levels are altered in vitiligo skin and peripheral blood mononuclear cells (PBMCs). Blocking NLRP3 signaling together with IL-12/23 inhibition this likely to inhibit the differentiation of Th17 cells into differentiate into IFN-γ$^+$IL-17$^+$ Th17 cells (= Th17.1) and finally into Th1-like exTh17 cells with high IFN-γ and low/no IL-17 production (=non-conventional Th1 cells). These non-conventional Th1 cells are emerging players in auto-immune disorders as they are not constrained by regulatory T cells and become largely glucocorticoid resistant.[1] Inhibition of Th17.1 inducing cytokines would shift the balance towards regulatory Th17 cells (Treg17) - which retain the capacity to produce IL-17 - and ultimately regulatory exTh17 cells with limited remaining capacity to produce IL-17.





Example of improvement of alopecia areata using ustekinumab (Guttman-Yassky et al. 2015). Other case series showed mixed results.

In vitiligo, alopecia areata and toxic epidermal necrolysis increased numbers of Th17 cells and elevated IL-17 levels have been reported. We have conducted the first-ever trial with IL-17 inhibition (secukinumab) in vitiligo and clearly documented that IL-17 inhibition was not effective.[2] Similar results were obtained in alopecia areata.[3] In further experiments, we demonstrated that increased levels of IFN-γ$^+$IL-17$^+$ Th17 cells (Th17.1) were the most likely reason that previous studies suggested an upregulated Th17 pathway. Targeting the plasticity of the Th17 pathway and thereby preventing the development of pathogenic IFNγ$^+$(ex)Th17 cells may, therefore, be a new treatment option. The plasticity of the Th17 pathway is increasingly recognized as an attractive target of autoimmune disorders since direct interference with the Th1 pathway is often associated with important long-term adverse events (e.g. skin cancer, infections…). Overwhelming evidence documents that the final steps leading to epidermal cell destruction are strongly regulated by IFN-γ.[4]

Research methodology and work plan.

PART 1: INVESTIGATING CHANGES IN IL-1β, IL-12, IL-18, IL-23 SIGNALING AND CELL SUBSETS ORIGINATING FROM THE TH17 PATHWAY.

- Aim 1.1: Clarification of the systemic changes in IL-1β, IL-12, IL-18, IL-23 signaling and the Treg/Treg17/Th17/Th17.1/Th1-like cell balance over time in patients with vitiligo, alopecia areata, and toxic epidermal necrolysis.

In this part, we will investigate the influence between NLRP3 activity, IL-12, and IL-23 levels on Th17 plasticity in patients with vitiligo, alopecia areata and toxic epidermal necrolysis. NLRP3 activity, IL-12/23, Th17 subsets, and the chemokine profile will be investigated according to the affected body surface area (BSA), the disease activity and compared with healthy controls. The main hypothesis is that the combination of NLRP3 activity and IL-12 signaling is crucial for the conversion of Th17 lymphocytes into Th1-like cells. Previously, our group demonstrated that Th17.1 cells are elevated during disease flares in vitiligo patients and converted into regulatory Th17 cells in stable phases. NLRP3 and IL-12 are driving factors for the development of non-classical Th1 cells by limiting the differentiation into regulatory Th17 lymphocytes (Figure right). Our preliminary findings point to the increased presence of NLRP3 expression in vitiligo and alopecia areata patients (vitiligo: n = 20, alopecia areata: 15 versus n = 20 healthy controls).

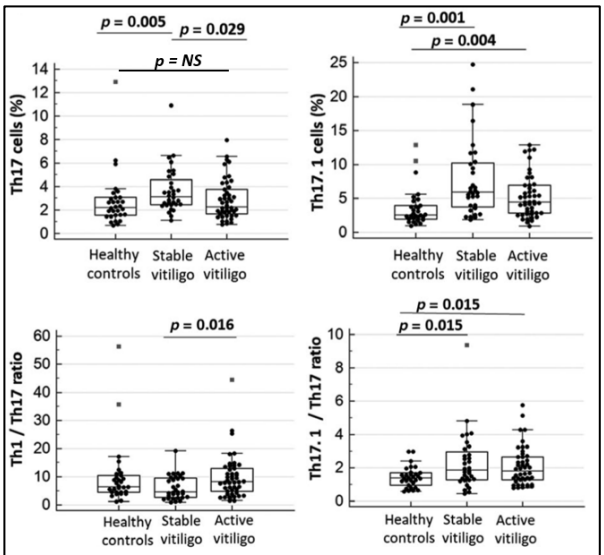


Figure: Th17 cells were not increased in active vitiligo patients, in contrast to Th17.1 cells. Both Th1/Th17 ratio and Th17.1/Th17 ratios were increased in patients with active vitiligo.

Study protocol:

Patients: Blood will be taken from 120 non-segmental vitiligo patients, 60 patients with alopecia areata and 30 patients with toxic epidermal necrolysis at 3 consecutive time points. These data will be compared with 50 healthy controls. For vitiligo patients, blood analysis will be done every 6 months, for alopecia areata patients every 3 months and in case of toxic epidermal necrolysis every 2 weeks. Peripheral blood mononuclear cells (PBMCs) will be collected by centrifugation on Ficoll-Paque and cryopreserved until analysis. Serum samples will also be stored. The strength of this part will be the large enrollment of patients and adequate follow-up time allowing inter- and intraindividual comparison at different time points. Moreover, a significant subset of vitiligo patients with alopecia areata will be recruited (n= 30). This ensures an important subset analysis.

This number of patients is based on a sample size calculation assuming a minimal 15% downregulation of NLRP3, IL-12 and IL-23 expression in vitiligo patients, alopecia areata patients and toxic epidermal necrolysis patients versus healthy controls (power: 0.8; two-sided α: 5%; standard deviation: 20% => n=26). The standard deviation is based on our previous immunohistochemistry experiments with NLRP3 and multiplex bead array of IL-1β, IL-12 and IL-23. Vitiligo patients will be enrolled during the specialized vitiligo consultations at our department (n=35 patients/week). Patients with alopecia areata and toxic epidermal necrolysis will also be enrolled at our department and several other departments (see section collaborations). To enhance the number of patients with toxic epidermal necrolysis both national and international university hospitals will be asked to participate.

For vitiligo, the disease activity will be measured using a modified Vitiligo Disease Activity score (VIDA) according to Njoo et al. 1999 and a clinical evaluation using the Vitiligo Signs of Activity Score (VSAS).[5,6] The affected body surface area of the vitiligo patients will be measured with the Vitiligo Extent Score (VES).[7] For alopecia areata, the disease extent will be measured by the Severity of Alopecia Tool II (SALT II) and disease activity by the Alopecia Areata Progressive Index.[8] The extent of toxic epidermal necrolysis will be measured by the 1% hand rule and the severity by the Severity-of-illness Score for Toxic Epidermal Necrolysis (SCORTEN).[9]

Methods: - ELISA analysis and multiplex bead array will be performed to assess the inflammasome activation by measuring caspase-1, IL-1β, IL-12, IL-18 and IL-23 on serum. On the other hand, mRNA analysis of PBMCs will be done for NLRP1 and NLRP3 expression.

- Regulatory T cells, regulatory Th17 cells, Th17, Th17.1, non-conventional Th1 and conventional Th1 subsets will be analysed according to their cytokine production and membrane markers. PBMCs will be stimulated with PMA/ionomycin and IL-17, IL-10 and IFN-γ production will be measured by flow cytometry. $CD3^+CD4^+Foxp3^+IL\text{-}17^+IL\text{-}10^+$ T cells will be classified as regulatory Th17 cells, $CD3^+CD4^+CD25^+Foxp3+$ as regulatory T cells, $CD3^+CD4^+IL\text{-}17^+IFN\gamma^-IL\text{-}10^-$ as Th17 cells, $CD3^+CD4^+IL17^+IFN\gamma^+$ cells as Th17.1 lymphocytes, $CD3^+CD4^+CD161^+IFN\gamma^+IL\text{-}17^-$ cells as Th1-like exTh17 lymphocytes and $CD3^+CD4^+CD161^-IFN\gamma^+IL\text{-}17^-$ as conventional Th1 cells.

- CXCL9, CXCL10, soluble CD25, sCD27 and S100B will be measured in serum as biomarkers of disease activity.

Statistical analyses: Median values between two groups will be compared by Student T-test or between >2 groups by One-way Anova (or Mann–Whitney U test/Kruskal–Wallis test in case of no normal distribution). To evaluate correlations, Spearman correlation coefficients will be calculated. All statistical analyses will be performed using SPSS 25.0 (SPSS Inc., Chicago, IL, USA)

- Aim 1.2: Investigate NLRP3, AHR, IL-12, IL-23 expression in the skin of vitiligo and alopecia areata and toxic epidermal necrolysis patients

- In this part, we will investigate whether NLRP3, IL-1β, IL-12, IL-18 and IL-23 expression are crucial in the collapse of the epidermal immune-privilege in vitiligo, alopecia areata, and toxic epidermal necrolysis. Subsequently, we will investigate whether these factors correlate with the lesional CD4 cells exhibiting a Th1 phenotype but originating from the Th17 lineage in vitiligo, alopecia areata, and toxic epidermal necrolysis. Biopsies will be taken from vitiligo lesions (non-lesional and perilesional), alopecia areata lesions (lesional, and non-lesional), and toxic epidermal necrolysis (lesional and non-lesional).

Methods: Biopsies of non-segmental vitiligo lesions (n=20 patients), halo naevi (n= 20), alopecia areata (n=20 lesional, n=20 non-lesional) and toxic epidermal necrolysis (n=20 lesional, n=20 non-lesional) will be taken.

Immunohistochemistry: The specimens for cryosections will be embedded, snap-frozen in liquid nitrogen and stored at -80C until use. Before staining, 7-µm thick sections will be cut. Immunohistochemistry of NLRP1, NLRP3, AHR, IL-1β, IL-12, IL-17, IL-23, CD4, CD8, IFNγ, RORγT, CD161, CXCL10, and MelanA will be performed.

RNA sequencing: Biopsies will be placed in QIAzol and homogenized using a TissueLyser. Total RNA will be extracted. mRNA analysis of NLRP1, NLRP3, AHR, CXCL9, CXCL10, RORγT, CD161, IFNγ, IL-10, IL-12, IL-17 and IL-23 will be performed using real-time qPCR analysis.

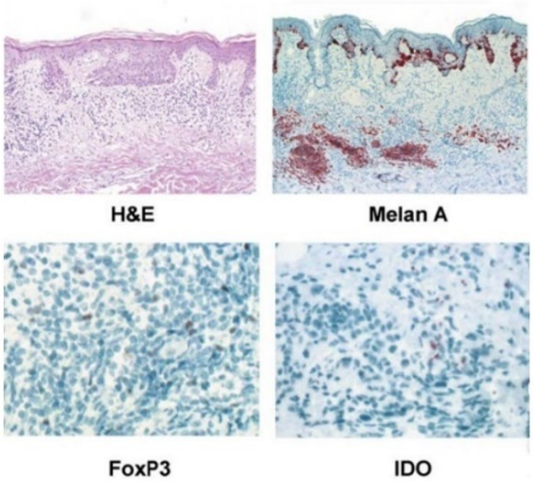


Figure: Our preliminary results in halo naevi and vitiligo point to a limited expression of IDO. This aligns with a downregulated aryl hydrocarbon receptor (AHR) pathway leading to lack of NLRP3 inhibition

Flow cytometry: skin lymphocytes will be isolated from biopsies of halo naevi, active vitiligo perilesional borders, alopecia areata skin, and toxic epidermal necrolysis according to Clark et al. 2006. Briefly, T cells will be isolated from short term skin explant cultures. The skin will be cut into small pieces and seeded upon Cellfoam matrices (Cytomatrix Pte LtD, Australia) after the matrices were incubated with a solution of 100 microgram/ml rat tail collagen I in phosphate-buffered saline (PBS) for 30 min at room temperature, followed by 2 rinses in PBS. Each matrix will be placed into Iscove's modified Dulbecco's medium with 20% heat-inactivated fetal bovine serum. Recombinant IL-2 and IL-15 will be added to stimulate lymphocyte activation and proliferation. The number of T helper cells originating from the Th17 lineage will be investigated using flow cytometry.

Power analysis: The number of 20 patients is based on an expected downregulation of at least 20% of mRNA expression between perilesional or lesional skin compared to non-lesional skin (power: 0.8; α = 5%, standard deviation: 20%).

Statistical analyses: Expression levels between lesional versus non-lesional skin will be compared using a paired two-samples t-test (or Wilcoxon signed-rank test in case of non-parametric data). NLRP3, IL-1β, IL-12, IL-18 and IL-23 expression will be correlated with the other above mentioned factors using Spearman's correlation to clarify the associated immunologic network.

Risk analysis and management part 1: This part carries a very low risk as most laboratory techniques have already been optimized at our lab. If recruitment would be slow, we have a network of international dermatology departments in the vitiligo field. These departments can also be contacted to recruit not only vitiligo patients but also patients with alopecia areata and toxic epidermal necrolysis.

PART 2: DETERMINING IF NRLP3, IL-12 AND IL-23 INFLUENCE TH17 PLASTICITY AND TH1-ASSOCIATED CHEMOKINE RELEASE.

- Aim 2.1: to study in vitro if the formation of Th17.1 cells and IFNγ$^+$exTh17 cells can be attenuated by inhibiting NLRP3 with or without IL-12 and/or IL-23 inhibition in a Th1 promoting environment.

- Th17 lymphocytes will be isolated from healthy donors using flow cytometry cell sorting and cultured for 21 days with an anti-CD3 antibody and recombinant IL-2. The first experiments will be carried out with different concentrations of tranilast (NLRP3 antagonist) and/or the aryl hydrocarbon receptor agonist tapinarof (= indirect NLRP3 inhibition via AHR) and supplemented to the supernatant to investigate its capacity to drive Th17 into a regulatory phenotype rather than a Th1 phenotype. If successful, further experiments will be conducted in combination with IL-12 and IL-23 inhibition. The generation of regulatory Th17 cells and IFN-γ producing Th17 cells will be measured.

- Blood will be drawn from vitiligo patients (patients from part 1) and the presence of anti-melanocyte specific T cells will be determined by HLA-A2/peptide tetramer staining against the melanocyte differentiation antigens Mart-

1, gp100, tyrosinase and a control antigen (influenza virus). We have previously optimized this tetramer staining procedure and our experiments confirm the increased presence of especially anti-MART-1 and anti-gp100-specific cytotoxic lymphocytes in vitiligo patients and patients with halo nevi. In patients with the highest number of melanocyte-specific T cells, these cells will be isolated using flow cytometry cell sorting. PBMCs of these patients will be cultured for 21 days with anti-CD3 antibody and recombinant IL-2 with and without the best combination for inhibiting TH17>Th1 plasticity of the previous experiment. This will simulate the effect of therapeutic inhibition of NLRP3 and IL-12/IL23 on the Th17 plasticity in a proinflammatory environment. CD4 lymphocytes originating from the Th17 pathway, CD3+CD4+CD161+ cells, will be isolated using flow cytometry cell sorting. CD3+CD4+CD161+ cells (5:1) exposed to NLRP3 + IL-12/IL23 inhibition will be added to the melanocyte-specific T-cells for 3 days. Subsequent stimulation with pooled melanocyte-specific peptides (tyrosinase$_{369-377}$, gp100$_{280-288}$, gp100$_{209-217}$ and MART-1$_{26-35}$) loaded on EBV-transformed B cells (JY) will be carried out. The production of IFN-ɣ, TNF-α, granzyme B and IL-17 by melanocyte-specific cytotoxic lymphocytes will be determined by flow cytometry and the production of IFN-ɣ, TNF-α, IL-10 and IL-17 will be measured in the supernatant by multiplex bead array. This will clarify if differences in the Th17 subsets induced by NLRP3 and IL-12/23 inhibition can impact the melanocyte specific CD8 T-cell response.

Risk analysis and management part 2: In case no difference in Th17 lineage derived cells would be observed, the focus will be shifted towards the direct influence of inhibiting NLRP3 and IL-12/23 on the activity of melanocyte-specific cytotoxic T cells. In that case, recombinant IDO or IDO transfected fibroblasts or dendritic cells will be added to the melanocyte-specific T-cells.

PART 3: INVESTIGATING IF TARGETING NLRP3 BY DIRECT OR AHR-DEPENDENT MECHANISMS ALONE OR IN COMBINATION WITH IL-12/23 INHIBITION IS PROMISING IN IFNγ-INDUCED SKIN DISORDERS.

- In this part, we will investigate if treatment to directly or indirectly (using AHR ligands) inhibit NLRP3 expression in combination with IL-12/23 inhibition is a promising strategy in vitiligo, alopecia areata and toxic epidermal necrolysis.

Methods: - Keratinocytes, melanocytes, and PBMCs from healthy controls and patients with vitiligo (n = 10), alopecia areata (n = 10), and toxic epidermal necrolysis (n = 10) will be primed with IFN-γ and/or CXCL10in combination with different concentrations of NLRP3 inhibition [tranilast (NLRP3 antagonist) and/or the aryl hydrocarbon receptor agonist tapinarof (= indirect NLRP3 inhibition via AHR] and IL-12/IL-23 inhibition (ustekinumab). This experiment will be repeated in combination with different ratios of NLRP3-transfected keratinocytes and melanocytes to investigate if the working mechanism of tranilast and tapinarof is indeed related to NLRP3 inhibition. The release of IFN-γ, IL-1β, IL-18, IL-12, IL-23, CCL3, CSF3, CXCL9, CXCL10, CXCL12, CCL5 and CCL20 chemokines will be investigated by ELISA and multiplex bead array. NLRP3 expression will be measured by immunohistochemical staining of NLRP3, IL-12, IL-23 on cytospin slides, by mRNA analysis and western blot. All experiments will be done in triplicate.

- To investigate the inhibiting capacity of the combination of methotrexate and ustekinumab on the cytokines involved in Th17>Th1 differentiation, skin cells and PBMCs will be subjected to different concentrations of All experiments will be done in triplicate. IFN-γ, IL-1β and IL-18 will be measured in the supernatant and by flow cytometry.

- Biopsies of perilesional (= vitiligo) and lesional skin (= alopecia areata and toxic epidermal necrolysis) will be treated with methotrexate, tranilast, tapinarof, ustekinumab and/or the strongest combination identified in part 2.1. CXCL10, IL-1-β, IL-12, IL-17, IL-23 and IFN-γ will be measured over time in the supernatant. After confirmation that IFN- γ activity can be inhibited, a skin explant model for vitiligo [n = 10 patients: for each patient 2 biopsies (= 1 treated and 1 control)] will be used according to van den Boorn et al. (2009) at AUMC, Amsterdam (see section collaborations). In this experiment, skin explants treated with NLRP3+IL-12/IL23 inhibition will be compared to untreated skin explants. NLRP3 expression and melanocyte destruction will be measured using immunohistochemistry with anti-NLRP3 antibodies, anti-gp100 and anti-active caspase 3 antibodies, respectively. The aim is to demonstrate that the combination of NLRP3 + IL-12/IL-23 inhibition can prevent the specific immune-mediated melanocyte attack in vitiligo.

Power analysis: A number of 10 x 2 skin explant models is based on an expected downregulation of at least 30% of difference in CXCL10 and IFN-γ expression in treated skin explants versus untreated skin explants (power: 0.8; α = 5%; standard deviation: 20%).

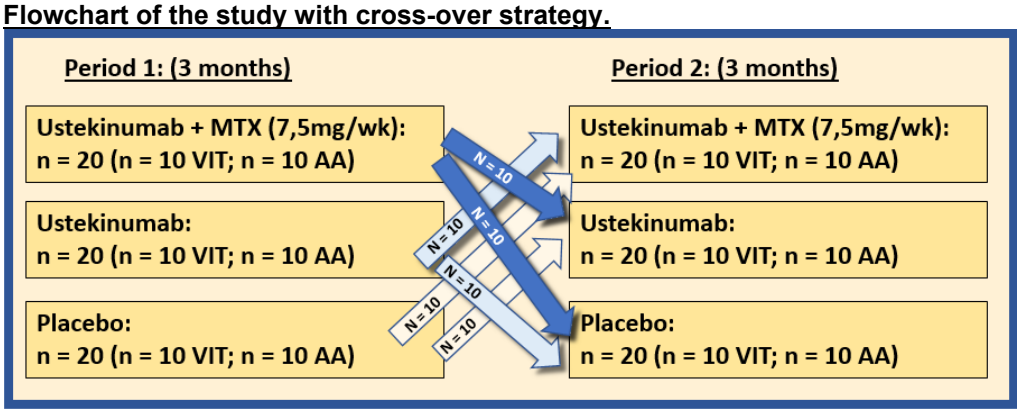
Statistical analyses: A comparison between two groups will be done using a T-test or in case of multiple groups with one-way ANOVA (in case of normally distributed data). Measurements over time will be analyzed with a paired-T test (in case of normally distributed data) or one-way repeated measure ANOVA. Correlation analysis will be done using the Spearman's rank correlation.

PART 4: TOWARDS A SYNERGISTIC APPROACH: INVESTIGATION IF A COMBINATION OF USTEKINUMAB WITH TOPICAL TRANILAST AND/OR SYSTEMIC METHOTREXATE HAS A SYNERGISTIC ACTION BY INHIBITING THE KEY CYTOKINES THAT DRIVE TH17 PLASTICITY.

- Methotrexate is an effective treatment for both vitiligo and alopecia areata. While higher dosages are usually required for alopecia areata, our observations showed that low maintenance dosages (7,5 mg/week) can lead to remarkable repigmentation in patients with vitiligo. A retrospective analysis of patients treated at our department with 7,5 mg/week methotrexate in combination with UVB showed 2-fold accelerated repigmentation times and 40% more repigmentation compared to monotherapy with UVB (manuscript in preparation). Remarkably, higher dosages do not lead to superior results. Methotrexate downregulates NLRP3 activation, IL-1β, IL-12 and IL-18 in patients with rheumatoid arthritis.[10] Treatment with ustekinumab (= IL-12/23 inhibitor) has resulted in clear responses in patients with alopecia areata and vitiligo, although contradictory results have been found. Our hypothesis is that combined inhibition of IL-12/23 in combination with a topical or systemic agent that inhibits IL-1β and IL-18 will result in more predictable effects. In this study we will investigate if combinatory inhibition with low-dose methotrexate has a synergistic effects in vitiligo and alopecia areata by inhibiting the key cytokines that drive Th17 plasticity. Ustekinumab will also be studies in combination with the topical NLRP3 inhibitor tranilast.

Methods: - 30 patients with non-segmental vitiligo and 30 patients with alopecia areata will be in included in this study. This study includes a cross-over strategy where different treatments (ustekinumab + methotrexate; ustekinumab monotherapy, and placebo) will be compared with a cross-over after 3 months (see flow chart). Additionally, all patients will be half-sided treated with topical tranilast (or other topical treatment with best performance based on the previous experiments). Inclusion criteria for non-segmental vitiligo patients will be age >18 years, progression in the last 3 months, affected BSA>3% and presence of lesions in the face. Inclusion criteria for alopecia areata will be age >18 years, active hair loss in the last 2 months and hair loss between 10-90% of the scalp.
In vitiligo patients, the evolution of depigmented areas will be scored using the VESplus and comparisons will be made between group A and group B. Alopecia areata will be scored using SALT II and manual tracing of the areas of hair loss with semi-automatic surface analysis using ImageJ (left versus right; AHR versus placebo).[8] Blood will be drawn every 2 months for 8 months to investigate the immune cell subsets originating from the Th17 lineage as outlined above. After 3 months, a biopsy will be taken in a perilesional progressing border of vitiligo and an area of active hair loss of alopecia areata identified at baseline. Immunohistochemistry will be performed for NLRP1, NLRP3, CD4, CD8, Natural killer group 2D (NKGD2D), CD45RA and CD69. AHR, IFNγ, RORγT, CXCL9, CXCL10, IL-1β, IL-18, TNF-α, IL-17, and IL-10 will be analyzed by real-time quantitative PCR.

Flowchart of the study with cross-over strategy.



Power analysis: The number of 20 patients in each group is based on an expected downregulation of at least 20% of mRNA expression between baseline and 3 months of treatment (power: 0.8; α = 5%, standard deviation: 20%).
Statistical analyses: will be performed according to aim 1.1 + paired comparisons will be analyzed with a paired-T test (or in case of not normally distributed data: Wilcoxon signed rank test). Correlation analysis will be done using the Spearman's rank correlation.
Risk analysis and management part 4: If the combination treatment of MTX with ustekinumab would no induce repigmentation after 3 months in patients with vitiligo, phototherapy (narrow band-UVB) will be added during the second part of the study (after cross-over). In the latter case, the timing of the biopsy will be changed from 3 months to 6 months.