ΕΘΝΙΚΟ ΚΑΙ ΚΑΠΟΔΙΣΤΡΙΑΚΟ ΠΑΝΕΠΙΣΤΗΜΙΟ

ΣΧΟΛΗ ΕΠΙΣΤΗΜΩΝ ΥΓΕΙΑΣ

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ΔΙΕΥΘΥΝΤΗΣ: ΔΗΜΗΤΡΙΟΣ ΒΑΣΙΛΟΠΟΥΛΟΣ

ΓΕΝΩΜΙΚΗ ΑΝΑΛΥΣΗ ΣΤΟ ΣΥΣΤΗΜΑΤΙΚΟ ΕΡΥΘΗΜΑΤΩΔΗ ΛΥΚΟ: ΣΤΟΧΕΥΜΕΝΗ ΑΝΑΛΥΣΗ ΤΟΥ ΜΕΤΑΓΡΑΦΩΜΑΤΟΣ ΓΙΑ ΔΙΑΓΝΩΣΗ ΚΑΙ ΕΞΑΤΟΜΙΚΕΥΜΕΝΗ ΘΕΡΑΠΕΙΑ

Γαραντζιώτης Παναγιώτης

ΔΙΔΑΚΤΟΡΙΚΗ ΔΙΑΤΡΙΒΗ

ΑΠΡΙΛΙΟΣ 2023

ΑΘΗΝΑ

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GENOMIC ANALYSIS OF SYSTEMIC LUPUS ERYTHEMATOSUS: TARGETED TRANSCRIPTIONAL ANALYSIS FOR DIAGNOSTICS AND PERSONALIZED IMMUNOTHERAPY

Garantziotis Panagiotis MD, MSc

DOCTORAL THESIS

APRIL 2023

ATHENS

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Γνωστικό αντικείμενο: Γενωμική ανάλυση στο Συστηματικό Ερυθηματώδη Λύκο: Στοχευμένη ανάλυση του μεταγραφώματος για διάγνωση και εξατομικευμένη θεραπεία

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1. **Δημήτριος Βασιλόπουλος**: Καθηγητής Παθολογίας – Ρευματολογίας
2. Δημήτριος Μπούμπας: Καθηγητής Παθολογίας – Ρευματολογίας
3. Γεώργιος Μπερτσιάς: Αναπληρωτής Καθηγητής Ρευματολογίας-Κλινικής Ανοσολογίας, Ιατρικής Σχολής Πανεπιστημίου Κρήτης

Α) Ημερομηνία αιτήσεως υποψηφίου: 18.10.2019

Β) Ημερομηνία ορισμού Τριμελούς Συμβουλευτικής Επιτροπής: 22.11.2019

Γ) Ημερομηνία Ορισμού Θέματος: 31.01.2020

Δ) Ημερομηνία έγκρισης Θέματος: 31.01.2020

Ε) Ημερομηνία κατάθεσης της διδακτορικής διατριβής: 03.02.2023

ΒΙΟΓΡΑΦΙΚΟ ΣΗΜΕΙΩΜΑ

Education and Medical Training

05/2023-dato
Medical Residency, Department of Rheumatology and Immunology, Erlangen University Hospital

10/2020-04/2023
Medical Residency, Department of Rheumatology and Immunology, Hannover Medical School (MHH)

- 6-month Rotation, Department of Nephrology
- 6-month Rotation, Emergency Department
- 6-month Rotation, Intensive Care Unit

02-10/2020
Research Fellow, **New Horizon Fellowship**, Department of Rheumatology and Clinical Immunology, Charité and DRFZ

- Title: **„Targeting CD38 with Daratumumab in Refractory Systemic Lupus Erythematosus"**
- Supervisor: Dr. med. Tobias Alexander

12/2019-dato
Ph.D. Candidate, School of Medicine, National and Kapodistrian University of Athens

- Title: **„Genomic analysis of systemic lupus erythematosus: Targeted transcriptional analysis for diagnostics and personalized immunotherapy"**
- Supervisor: Prof. Dr. Dimitrios Vasilopoulos, Prof. Dr. Dimitrios Boumpas, Prof. Dr. George Bertsias

07/2018- 1/2020
Research Fellow, Rheumatology and Immunology, 4th Department of Medicine, National and Kapodistrian University of Athens Medical School, Athens

- Director: Prof. Dr. Dimitrios Boumpas

10/2017-08/2019
M.Sc. „Molecular Biomedicine: Mechanisms of Disease, Molecular and Cellular Therapies, and Bioinnovation", School of Medicine, National and Kapodistrian University of Athens

- Title: **„Whole blood transcriptomic analysis in ANCA associated vasculitis"**
- Supervisor: Prof. Dr. Dimitrios Boumpas
- *Cum laude*

09/2010-07/2016
Medical Degree, National and Kapodistrian University of Athens

- *Cum laude*

Publications

Ostendorf L, Burns M, Durek P, Heinz GA, Heinrich F, **Garantziotis P**, Enghard P, Richter U, Biesen R, Schneider U, Knebel F, Burmester G, Radbruch A, Mei HE, Mashreghi MF, Hiepe F, Alexander T. Targeting CD38 with Daratumumab in Refractory Systemic Lupus Erythematosus. **N Engl J Med**. 2020 Sep 17;383(12):1149-1155. doi: 10.1056/NEJMoa2023325. PMID: 32937047.

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Frangou E, **Garantziotis P**, Grigoriou M, Banos A, Nikolopoulos D, Pieta A, Doumas S , Fanouriakis A , Hatzioannou A, Manolakou A, Alissafi T, Verginis P, Athanasiadis E, Dermitzakis E, Bertsias G, Filia A, Boumpas D. Cross-species transcriptome analysis for early detection and specific therapeutic targeting of human lupus nephritis. **Ann Rheum Dis.** 2022 Jul 29:annrheumdis-2021-222069. doi: 10.1136/annrheumdis-2021-222069. Epub ahead of print. PMID: 35906002.

Banos A*, Thomas K*, **Garantziotis P***, Filia A, Malissovas N,Pieta A, Nikolakis D, Panagiotopoulos AG, Chalkia A, Petras D, Bertsias G, Boumpas DT and Vassilopoulos D.The genomic landscape of ANCA-associated vasculitis: Distinct transcriptional signatures, molecular endotypes and comparison with systemic lupus erythematosus. **Front. Immunol.** 2023 14:1072598 doi: 10.3389/fimmu.2023.1072598 (***Equal contribution**)

Nikolakis D*, **Garantziotis P***, Sentis G, Fanouriakis A, Bertsias G, Frangou E, Nikolopoulos D, Banos A, Boumpas DT. Restoration of Aberrant Gene Expression of Monocytes in Systemic Lupus Erythematosus via a Combined Transcriptome-Reversal and Network-Based Drug Repurposing Strategy. **BMC Genomics**. 2023 (Accepted) (*** Equal contribution**)

Doumas SA, Tsironis C, Bolaji AA, **Garantziotis P**, Frangou E. Glomerulonephritis and inflammatory bowel disease: A tale of gut-kidney axis dysfunction. **Autoimmun Rev.** 2023 Mar 28;22(6):103327. doi: 10.1016/j.autrev.2023.103327. PMID: 36990134.

Nezos A, Skarlis C, Psarrou A, Markakis K, **Garantziotis P**, Papanikolaou A, Gravani F, Voulgarelis M, Tzioufas AG, Koutsilieris M, Moutsopoulos HM, Kotsifaki E, Mavragani CP. Lipoprotein-Associated Phospholipase A2: A Novel Contributor in Sjögren's Syndrome-Related Lymphoma? **Front Immunol**. 2021 Jun 18;12:683623. doi: 10.3389/fimmu.2021.683623. PMID: 34220834; PMCID: PMC8253309.

Grivas A, Fragoulis G, **Garantziotis P**, Banos A, Nikiphorou E, Boumpas D. Unraveling the complexities of psoriatic arthritis by the use of -Omics and their relevance for clinical care. **Autoimmun Rev**. 2021 Nov;20(11):102949. doi: 10.1016/j.autrev.2021.102949. Epub 2021 Sep 10. PMID: 34509654.

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Gergianaki I, **Garantziotis P**, Adamichou C, Saridakis I, Spyrou G, Sidiropoulos P, Bertsias G. High Comorbidity Burden in Patients with SLE: Data from the Community-Based Lupus Registry of Crete. **J Clin Med**. 2021 Mar 2;10(5):998. doi: 10.3390/jcm10050998. PMID: 33801229; PMCID: PMC7957898.

Lamprianidou E, Kordella C, Kazachenka A, Zoulia E, Bernard E, Filia A, Laidou S, **Garantziotis P**, Vassilakopoulos TP, Papageorgiou SG, Pappa V, Galanopoulos AG, Viniou N, Nakou E, Kalafati L,

Chatzidimitriou A, Kassiotis G, Papaemmanuil E, Mitroulis I, Kotsianidis I. Modulation of IL-6/STAT3 signaling axis in CD4+FOXP3- T cells represents a potential antitumor mechanism of azacitidine. **Blood Adv**. 2021 Jan 12;5(1):129-142. doi: 10.1182/bloodadvances.2020002351. PMID: 33570632; PMCID: PMC7805308.

Nikolopoulos D, Kitsos D, Papathanasiou M, Chondrogianni M, Theodorou A, **Garantziotis P**, Pieta A, Doskas T, Bertsias G, Voumvourakis K, Boumpas DT, Fanouriakis A. Demyelination with autoimmune features: a distinct clinical entity? Results from a longitudinal cohort. **Rheumatology (Oxford)**. 2021 Sep 1;60(9):4166-4174. doi: 10.1093/rheumatology/keaa902. PMID: 33404657.

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Adriawan IR, Atschekzei F, Dittrich-Breiholz O, **Garantziotis P**, Hirsch S, Risser LM, Kosanke M, Schmidt RE, Witte T, Sogkas G. Novel aspects of regulatory T cell dysfunction as a therapeutic target in giant cell arteritis. **Ann Rheum Dis**. 2022 Jan;81(1):124-131. doi: 10.1136/annrheumdis-2021-220955. Epub 2021 Sep 28. PMID: 34583923; PMCID: PMC8762021.

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Ad hoc Reviewer

Frontiers in the Immunology, Journal of Clinical Medicine, Autoimmunity

Oral presentations

Garantziotis P., Nikolakis D., Doumas S., Fragou E., Fanouriakis A., Filia A., Witte T., Bertsias G., Boumpas D. **„DEFINING SYSTEMIC LUPUS ERYTHEMATOSUS MOLECULAR TAXONOMY THROUGH DATA-DRIVEN RESTRATIFICATION AND IDENTIFICATION OF CLUSTER-TAILORED DRUGS FOR A PERSONALIZED MEDICINE APPROACH"**

- EULAR 2021, Annual European Congress of Rheumatology

Banos A., Thomas K., Malisiovas N., Filia A., **Garantziotis P.**, Makri A., Petras A., Nikolopoulos D., Pieta A., Katsimbri P., Bertsias G., Boumpas D., Vasilopoulos D. **„Transcriptomic analysis in ANCA associated vasculitis using RNA-sequencing"**

- Congress of Rheumatology, December 06-09/2018, Greek Rheumatology Society
- Award

Nikolopoulos D., Pieta A., Kostopoulou M., **Garantziotis P**., Katsimbri P., Boumpas D., Fanouriakis A. **„Stroke in SLE: Cohortstudy „Attikon"**

- Congress of the medical society of Athens, May 15-18/2019

Posters

Manolakou T., Tsiara I., Nikolopoulos D., **Garantziotis P.**, Benaki D, Gikas E., Frangou E., Mikroa E., Boumpas

D. **„Combined Analysis of Metabolic and Transcriptomic Kidney Profiles of NZW/B-F1 Murine Lupus Uncovers Biological Mechanisms Preceding the Onset of nephritis"**

- EULAR 2020, Annual European Congress of Rheumatology

Frangou E., **Garantziotis P.**, Grigoriou M., Banos A., Panousis N., Dermitzakis E., Bertsias G, Boumpas D, Filia A. **„COMPARATIVE TRANSCRIPTOME ANALYSES ACROSS TISSUES AND SPECIES IDENTIFY TARGETABLE GENES FOR HUMAN SYSTEMIC LUPUS ERYTHEMATOSUS (SLE) AND LUPUS NEPHRITIS (LN)"**

- EULAR 2020, Annual European Congress of Rheumatology

Ostendorf L., **Garantziotis P**., Wagner D., Durek P., Heinrich F., Enghard P., Burmester G., Radbruch A., Mashreghi M., Hiepe F., Alexander T. **„CD38+ MEMORY T CELLS ARE A FUNCTIONALLY DISTINCT SUBSET THAT IS EXPANDED IN SLE AND ASSOCIATED WITH LUPUS NEPHRITIS"**

- EULAR 2021, Annual European Congress of Rheumatology

Licensure

06/2019	**Medical License, Germany**
08/2016	**Medical License, Greece**

Internship

09/2014	Innere Medizin III (Kardiology), University Hospital Heidelberg, Germany
9/2015	Department of Haematology, University Hospital Manchester, UK
09/2016-07/2017	Mandatory Military Service, Physician, Hellinic Navy

Awards-Scholarships

2020	„New Horizon Fellowship", Charité
2018	Award for the best Oral presentation, Congress of Rheumatology, December 06-09/2018, Greek Rheumatology Society
2010	Eurobank EFG Scholarship: Award for excellent performance in the University entrance exams, Glyfada, Athens

Foreign Language

Deutsch	Goethe-Certificate C1
English	Certificate of Proficiency in English (C2 Cambridge University, UK) Certificate of Proficiency in English (C2 University of Michigan, US)

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ΙΑΥΤΑ. ΟΡΚΟΝ ΜΕΝ ΟΥΝ ΜΟΙ ΤΟΝΔΕ ΕΠΙΤΕΛΕ
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ΕΥΧΑΡΙΣΤΙΕΣ

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Θα ήθελα να ευχαριστήσω τους φίλους, την οικογένειά μου και ιδιαίτερα τη μητέρα μου για τη στήριξη και την κατανόηση.

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Abstract

Systemic Lupus erythematosus (SLE) is a complex, systemic autoimmune disease that can affect multiple organs either simultaneously or sequentially. Despite growing understanding of the disease driving mechanisms, diagnosis is primarily clinical und treatment remains empiric and for a significant number of patients, is far from being optimal. Current classification criteria as well as the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) score do not predict disease prognosis and treatment responses. Moreover, the highly heterogeneous clinical presentation of the SLE coupled with the diversity of abnormalities that have been elucidated at cellular and molecular level have accounted for the modest results of several SLE clinical trials. Patients with SLE are in need for early diagnosis and a molecular based patient stratification to guide targeted therapy.

In our study, we stratified patients with SLE, according to their distinct, whole blood molecular fingerprints, irrespective of their clinical annotation. To this end, we analyzed the peripheral blood transcriptional profiles of 120 patients with moderate to severe SLE. By applying a co-expression network analysis, we identified groups of transcripts (modules) that present common patterns of expression and we examined the enrichment of each module in the transcriptome of each patient, separately. Next, using agglomerative hierarchical clustering, based on the enrichment of each gene module, we determined patients' molecular endotypes. We identified a "Neutrophil" signature group, which almost exclusively comprised of patients with active Lupus Nephritis, whereas humoral and type I interferon responses were predominantly enriched in the "B cell" group. Macroautophagy disturbances, deregulation of pathways involved in toll-like receptor (TLR) and abberancies in mitochondrial function distinguished the "Autophagy" and "Metabolism" groups, respectively. Lastly, platelet activation and hemostasis pathways characterized the "Hemostasis" group. Next, using the patient endotype specific signatures as input and leveraging one of the largest drug signature databases to date, iLINCS, we constructed an *in-silico*, signature-based drug prediction pipeline in order to propose compounds that are predicted to reverse the patients' transcriptional disturbances most effectively, in a personalized manner. Bortezomib was predicted to counteract the transcriptional changes of the patients of the "Neutrophil" group most efficiently. The patients of the "B-cell" endotype might benefit most from a treatment with azathioprine and ixazomib, whereas fostamatinib might represent a putative therapeutic option for patients of the "Metabolism" group.

To identify novel therapeutic SLE agents that might target the endotype-specific transcriptional disturbances, we performed a personalized drug repurposing analysis. Taking a step forward, applying two independent - a transcriptome-reversal and a network-based - strategies, we proposed compounds that might remedy transcriptional disturbances of monocytes in SLE. Finally, applying a cross-species, time-series transcriptional analysis, we determined a unifying mouse-kidney specific gene signature, which could predict with high accuracy patients that will develop Lupus Nephritis.

Περίληψη

Ο Συστηματικός Ερυθηματώδης Λύκος (ΣΕΛ) είναι ένα πολύπλοκο, αυτοάνοσο νόσημα, που μπορεί να προσβάλει πολλαπλά όργανα. Παρά τη σημαντική πρόοδο στην κατανόηση των μηχανισμών του νοσήματος, η διάγνωση είναι κλινική και η απόκριση στη θεραπεία παραμένει εμπειρική και για σημαντικό αριθμό ασθενών μη ικανοποιητική. Ενώ τα ισχύοντα κριτήρια ταξινόμησης της νόσου και ο δείκτης SLEDAI, αδυνατούν να προβλέψουν την πρόγνωση ή την ανταπόκριση στη θεραπεία, υπογραμμίζοντας την επιτακτική ανάγκη για τη μοριακή ταξινόμηση των ασθενών με ΣΕΛ. Η εκσεσημασμένη ετερογένεια των κλινικών εκδηλώσεων σε συνδυασμό με την ποικιλία διαταραχών που έχουν αναγνωριστεί σε κυτταρικό και μοριακό επίπεδο ευθύνονται για αρνητικά αποτελέσματα πολλών κλινικών μελετών στο ΣΕΛ.

Στη μελέτη μας, ταξινομήσαμε τους ασθενείς με ΣΕΛ, σύμφωνα με τα διακριτά τους μοριακά αποτυπώματα στο περιφερικό αίμα, ανεξάρτητά από τις κλινικές τους εκδηλώσεις. Ειδικότερα, αναλύσαμε τις μεταγραφικές υπογραφές του περιφερικού αίματος 120 ασθενών με μέτριο ή σοβαρό ΣΕΛ. Εφαρμόζοντας ανάλυση δικτύων συνέκφρασης γονιδίων, προσδιορίσαμε ομάδες γονιδίων (module) που εμφανίζουν κοινά πρότυπα έκφρασης και εξετάσαμε το βαθμό έκφρασης της κάθε ομάδας γονιδίων στο μεταγραφώμα του κάθε ασθενούς. Στη συνέχεια, χρησιμοποιώντας ιεραρχική συσσωρευτική μέθοδο ταξινόμησης, προσδιορίσαμε μοριακούς ενδοτύπους ασθενών με βάση το βαθμό έκφρασης της κάθε ομάδας γονιδίων. Εντοπίσαμε τον ενδότυπο "Ουδετερόφιλο", ο οποίος σχεδόν αποκλειστικά περιελάμβανε ασθενείς με ενεργό νεφρίτιδα Λύκου, ενώ υπογραφές χυμικής ανοσίας και ιντερφερόνης τύπου Ι προεξάρχουν στον ενδότυπο "Β κύτταρο". Παρουσία γονιδιακών υπογραφών ενδεικτικών δυσλειτουργίας των μιτοχονδρίων αποτελεί διακριτό χαρακτηριστικό του ενδοτύπου "Μεταβολισμός". Διαταραχές των μονοπατιών της μακροαυτοφαγίας και της σηματοδότησης μέσω τύπου Toll υποδοχέων ήταν ενδεικτικές του ενδότυπου "Αυτοφαγία". Τέλος, υπογραφές ενεργοποίησης των αιμοπεταλίων και αιμόστασης χαρακτήριζαν τον ενδότυπο "Αιμόσταση". Στη συνέχεια, χρησιμοποιώντας τη γονιδιακή υπογραφή κάθε ενδοτύπου ως βάση και εκμεταλλευόμενοι μια από τις μεγαλύτερες βάσεις γονιδιακών υπογραφών φαρμάκων (iLINCS), αναπτύξαμε έναν υπολογιστικό αλγόριθμο πρόβλεψης θεραπείας, με σκοπό να προτείνουμε θεραπείες που αντιστρέφουν εξατομικευμένα τις μεταγραφικές διαταραχές των ασθενών. Η βορτεζομίμπη ενδεχομένως αντιστρέφει πιο αποτελεσματικά τις μεταγραφικές διαταραχές των ασθενών του ενδότυπου "Ουδετερόφιλο". Οι ασθενείς που ανήκουν στον ενδότυπο "Β κύτταρο"

ενδεχομένως ωφελούνται περισσότερο από θεραπεία με την αζαθειοπρίνη ή ιξαζομίμπη, ενώ η φοσταματινίμπη μπορεί να θεωρηθεί θεραπευτική επιλογή για τους ασθενείς του ενδοτύπου “Μεταβολισμός”.

Προκειμένου να προτείνουμε νέες θεραπείες του ΣΕΛ, που ενδεχομένως στοχεύουν τις μεταγραφικές υπογραφές του κάθε ενδοτύπου, πραγματοποιήσαμε μία εξατομικευμένη μελέτη επαναστόχευσης φαρμάκων. Εφαρμόζοντας δύο ανεξάρτητες – μία αναστροφής μεταγραφικής υπογραφής και μία βασισμένη στο μοριακό δίκτυο- στρατηγικές επαναστόχευσης φαρμάκων, προτείναμε θεραπείες που ενδεχομένως αντιστρέφουν τις μεταγραφικές διαταραχές των μονοκυττάρων στο ΣΕΛ.

Τέλος, εφαρμόζοντας μία συνδυασμένη μεταγραφική ανάλυση στο ποντικό και τον άνθρωπο, προσδιορίσαμε μία γονιδιακή υπογραφή ενδεικτική νεφρίτιδας Λύκου στο πειραματικό μοντέλο ποντικού για ΣΕΛ, η οποία προβλέπει με ακρίβεια την ανάπτυξη νεφρίτιδας Λύκου.

Introduction

Diagnosis and management of SLE

Systemic Lupus Erythematosus (SLE) is a prototypic autoimmune disease that can have devastating effects on various organs including the kidneys, the skin, the joints, and the central nervous system and is defined by the aberrant production of antinuclear antibodies (ANA) [**1**]. SLE is a global disease, with the annual incidence rate in Europe ranging from 1 to 4.9 per 100,000 [**2-4**]. SLE predominantly affects women of childbearing age [**2**] and is among the leading causes of death in females of this age group [**5**]. Racial and ethnic disparities characterize prevalence, severity, and clinical course of SLE [**2**]. Patients with SLE of African and Hispanic ancestry are at higher risk for SLE associated renal involvement and suffer from significantly increased SLE related mortality compared with Caucasians [**2**].

Clinical heterogeneity defines SLE. The major clinical features and their frequency are illustrated in **Figure 1** [**6**]. SLE is a largely clinical diagnosis -supported by laboratory findings- after excluding alternative diagnoses. A diagnostic approach to patients with suspected SLE, incorporating a combination of the ACR-1997, SLICC-2012 and EULAR/ACR-2019 classification criteria, has previously been proposed by our group (**Figure 2.**) [**6**].

The clinical spectrum of SLE encompasses several distinct endotypes. SLE with antiphospholipid syndrome constitutes an evolving SLE phenotype, which displays increased risk of neuropsychiatric SLE (NPSLE), thrombotic and obstetric complications [**6**, **7**]. Although childhood-onset SLE (cSLE) is a rare disease, it has captured much attention due to its impact on the growth and development of the affected individuals [**6**]. Patients with cSLE more frequently suffer from severe disease and are more likely to experience high disease activity at presentation [**6**]. Albeit SLE is considered a mainly multisystem autoimmune disease, organ-dominant disease courses (musculoskeletal, dermatologic, haematologic, renal, neurological) can also occur, often complicating accurate diagnosis.

SLE is typically a relapsing-remitting disease, whereas long quiescent and chronic active disease patterns account for 30% of SLE cases [**6**, **8**]. Achieving remission is associated with reduced damage accrual in SLE [**9**]. The remission rates of SLE from large published series widely vary, while the highest prevalence of prolonged remission among Caucasian patients with SLE was observed in an italian cohort with 37% [**6**, **10**]. Adverse prognostic factors

related to unremitting disease included the haematological manifestation and the glomerulonephritis [**10**].

Accurate assessment of the disease activity as well as clear definition of response criteria and disease states are fundamental, as they largely guide clinical practice in SLE. Several activity indices -validating global or organ specific disease activity- have been proposed, including the SLE Disease Activity Index (SLEDAI), the British Isles Lupus Activity Group (BILAG) index, the Safety of Estrogens in Lupus Erythematosus National Assessment (SELENA)-SLEDAI Physician Global Assessment (PGA) and the SLE Disease Activity Score (SLE-DAS) [**6**, **11**]. Notably, the current activity indices exhibit limitations affecting the success of SLE clinical trials. To this end, the investigators in the belimumab trials employed the SLE Responder Index (SRI), which comprises criteria from three different validated indices, the (SELENA)-SLEDAI, the PGA and the BILAG [**6**]. An important step towards a treat-to-target strategy for SLE, was taken through the development of a consensus-based definition of remission (**Table 1.**) and lupus low- disease activity state (LLDAS) (**Table 2.**) [**12**-**14**].

Disease activity should be differentiated from damage, which is consistently associated with poor clinical outcomes and negatively affects survival of SLE patients [**6**, **15**]. The SLICC/ACR Damage Index (SDI) represents a widely used index to ascertain accumulation of organ damage in SLE, due to SLE itself, treatment complications or related comorbidities [**6**].

Despite the advances in the understanding of the SLE pathogenesis over the past decades, management of lupus patients poses challenges (**Figure 3.**). Attainment of sustained low disease activity state or remission and prevention of flares are the major therapeutic goals for patients with SLE. The antimalarial hydroxychloroquine is the mainstay long treatment in SLE, unless contraindications exist. Glucocorticoids remain a cornerstone of SLE treatment, however cumulative glucocorticoid exposure may lead to organ damage in patients with SLE [**16**]. To this end, glucocorticoid dose should not exceed 7.5 mg/day (prednisone equivalent), during chronic maintenance treatment [**6**]. To enable a more rapid tapering of glucocorticoids and reduce flare rates, prompt initiation of immunomodulatory agents, such as azathioprine, methotrexate, mycophenolate, cyclosporine is recommended [**6**]. Cardinal disease manifestation, childbearing potential and cost play a crucial role in the selection of the most appropriate immunomodulatory agent for each patient. For instance, patients with predominantly mucocutaneous and musculoskeletal features might benefit more from a treatment with methotrexate, whereas azathioprine or cyclosporine might be more suitable in haematological disease or when pregnancy is expected [**6**]. Cyclophosphamide and rituximab

are reserved for the treatment of life-, organ-threatening, refractory disease. Belimumab is a human monoclonal antibody that inhibits the soluble B lymphocyte stimulator (BLyS) and is the first biological agent approved by the U.S. Food and Drug Administration (FDA) for treatment of adults with persistently active or flaring SLE who are receiving standard therapy [**6**]. Efficacy of anifrolumab - a human monoclonal antibody to the type I IFN receptor subunit 1- across multiple organ domains was suggested by a post-hoc analysis of phase 3 TULIP-1 and TULIP-2 clinical trials, leading to the FDA approval for treatment of adult patients with moderate to severe, active, autoantibody-positive SLE. Interestingly, multitarget treatment represents an emerging therapeutic concept in SLE over the last decade. The AURA-LN phase 3 study has shown that the addition of the calcineurin inhibitor voclosporin to the standard of care induction therapy for Lupus Nephritis (LN) increased the rate of renal response, though more serious adverse events were observed [**17**]. Accordingly, the BLISS-LN study demonstrated that in active LN the combination therapy of belimumab with standard therapy was superior -in terms of renal responses- to standard therapy alone [**18**].

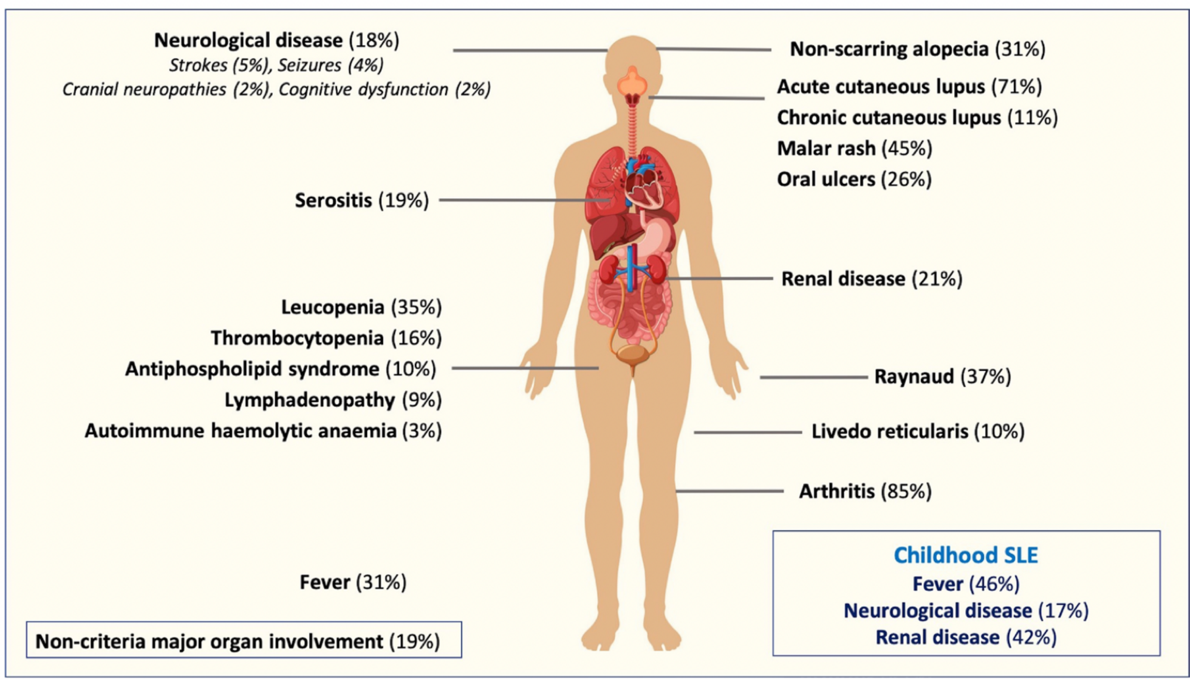

Figure 1. Clinical features of SLE and their frequency. Adopted by Fanouriakis A, Tziolos N, Bertsias G, *et al.*, 2021.

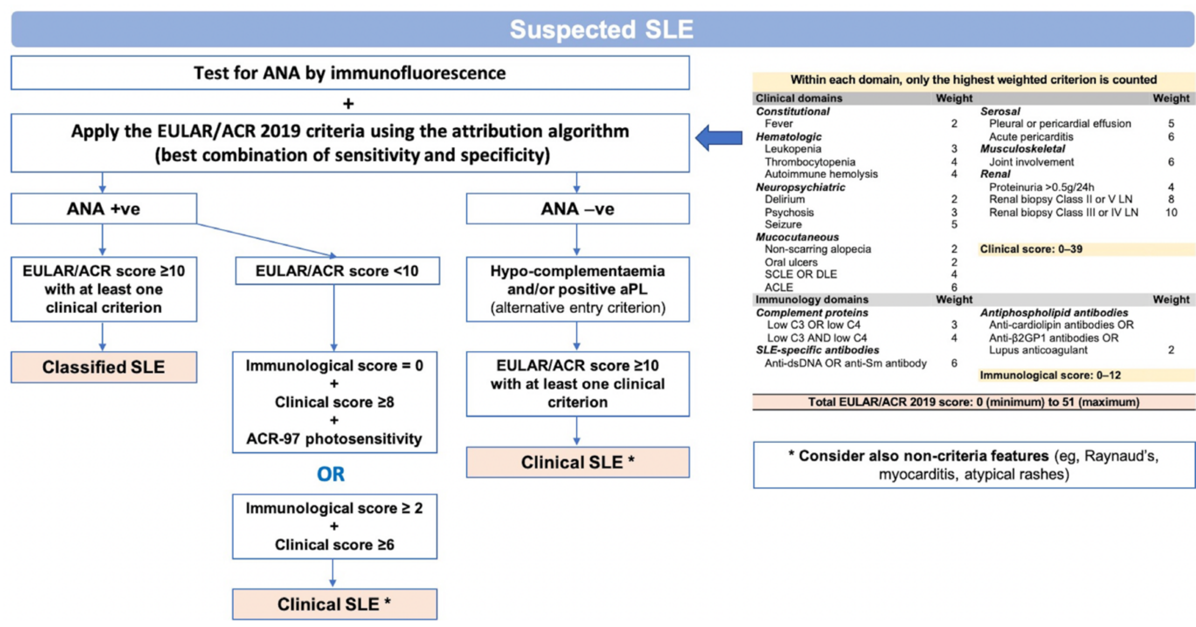

Figure 2. A diagnostic approach to patients with suspected SLE, incorporating a combination of the ACR-1997, SLICC-2012 and EULAR/ACR-2019 classification criteria. Adopted by Fanouriakis, *et al.*, 2020.

Definition of remission in SLE
For defining remission in SLE, a validated index must be used • Suggested indices are: clinical SLEDAI=0; BILAG 2004 D or E only; clinical ECLAM=0 These must be supplemented by the physician's global assessment being below an appropriate threshold (eg, <0.5 on a 0–3 scale)
A distinction will be made between remission off therapy and remission on therapy • Remission off therapy requires the patient to be on no other treatment for SLE than maintenance antimalarials Remission on therapy allows patients to be treated with maintenance antimalarials, stable, low-dose glucocorticoids (eg, prednisone ≤5 mg/day), maintenance immunosuppressives and/or stable (maintenance) biologics

Table 1. Definitions of remission in SLE. ECLAM, European consensus lupus outcome measure. Adopted by van Vollenhoven R, Voskuyl A, Bertsias G, *et al.*, 2017.

Definition of Lupus Low Disease Activity State
1. SLEDAI-2K ≤4, with no activity in major organ systems (renal, CNS, cardiopulmonary, vasculitis, fever) and no haemolytic anaemia or gastrointestinal activity
2. No new features of lupus disease activity 4.7 compared with the previous assessment
3. SELENA-SLEDAI physician global assessment (PGA, scale 0–3) ≤1
4. Current prednisolone (or equivalent) dose ≤7.5 mg daily
Well tolerated standard maintenance doses of immunosuppressive drugs and approved biological agents, excluding investigational drugs

Table 2. Lupus Low Disease Activity State definition. CNS, central nervous system; LLDAS, Lupus Low Disease Activity State; SLEDAI, Systemic Lupus Erythematosus Disease Activity Index. Adopted by Franklyn K, Lau CS, Navarra SV, *et al.*, 2015.

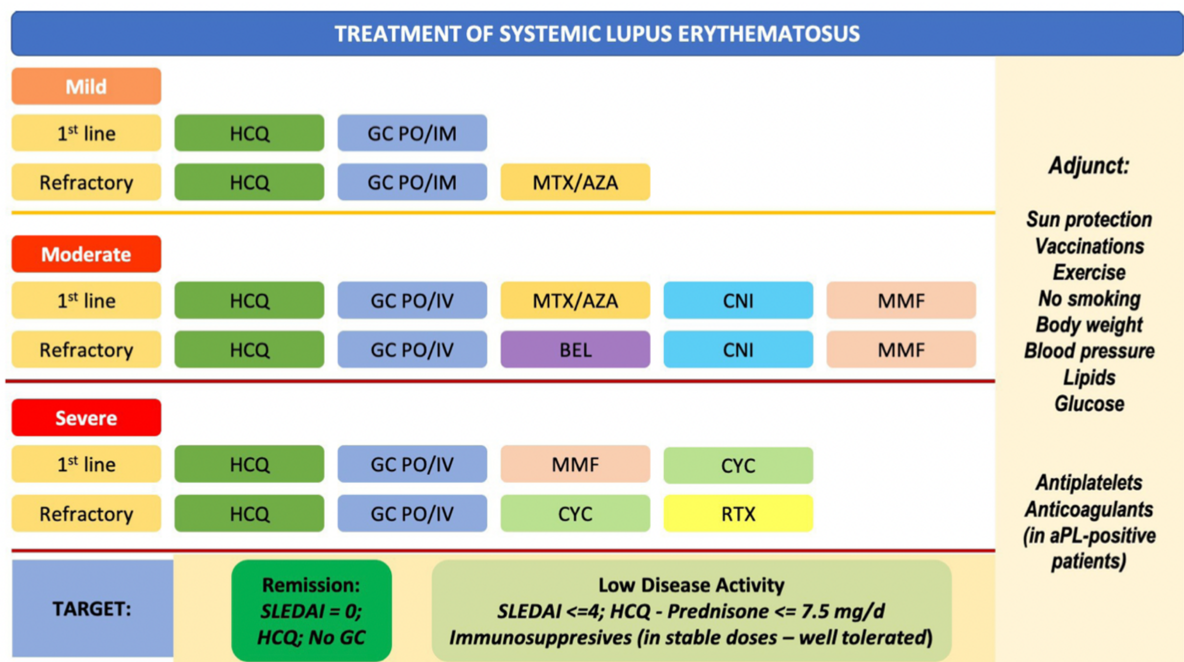

Figure 3. EULAR recommendations for the SLE treatment strategy and therapeutic goals. aPL, antiphospholipid antibody; AZA, azathioprine; BEL, belimumab; CNI, calcineurin inhibitors; CYC, pulse cyclophosphamide; EULAR, European League Against Rheumatism; GC, glucocorticoids; HCQ, hydroxychloroquine; MMF, mycophenolate mofetil; RTX, rituximab; SLEDAI, SLE Disease Activity Index. Adopted by Fanouriakis A, Tziolos N, Bertsias G, et al., 2021.

Aetiology and pathogenesis

Genetics and transcriptomics

The genetic contribution to the development of SLE is evident from the considerably high heritability (43.9%) among first degree relatives of patients with SLE [**19**]. Although several monogenic conditions - including single gene defects of complement component 1q (C1q) subcomponent A (C1QA), three-prime repair exonuclease 1 (TREX1), or deoxyribonuclease 1-like 3 (DNASE1L3) - can lead to SLE-like disease [**20**], in most cases a diverse array of genetic variants influence susceptibility to the disease.

Large genome-wide association studies (GWAS) have enabled the identification of risk alleles for SLE in or near genes linked to apoptotic mechanisms, DNA repair and clearance of cellular debris (TREX1, DNASE1, autophagy related 5 (ATG5)) [**19**]. SLE associated loci coding for proteins implicated with nucleic acid sensing machinery and type I interferon (IFN) signaling, such as interferon regulatory factor 5 (IRF5), signal transducer and activator of transcription 4 (STAT4), Toll-like receptor 7 (TLR7), and TLR9 have also emerged as putative disease genes [**19**]. Additionally, genetic variants within or near genes involved in B and T cell function, such as protein tyrosine phosphatase 22 (PTPN22), tumor necrosis factor superfamily member 4 (TNFSF4), protein phosphatase 2 catalytic subunit a (PPP2CA), B cell scaffold protein with ankyrin repeats 1 (BANK1), and cluster of differentiation 3ζ (CD3Z) loci might contribute to the T and B cell hyperactivity in SLE [**19**].

Most SLE genetic variants localize to non-coding, regulatory genomic regions and could thus determine the epigenetic dysfunction in SLE, with potential impact on the gene expression. Transcriptome analysis of the peripheral blood mononuclear cells (PBMCs) from SLE patients showed aberrant expression of genes related to type I IFN signaling and granulopoiesis [**21**, **22**]. Accordingly, Banchereau et al demonstrated an incremental enrichment of the neutrophilic gene expression signatures towards progression to active LN [**23**]. Taking a step forward, applying an unbiased clustering approach, the same group detected transcriptome modules associated with dysregulated natural killer (NK), T and B cell responses as well as a plasmablast signature indicative of active disease status [**23**]. In addition to gene expression signatures linked to adaptive and innate immunity, Panousis et al identified a whole blood "activity signature" enriched in immune cell metabolism, protein synthesis and proliferation pathways [**24**]. Notably, targeted transcriptional analysis of T cells

from the peripheral blood of SLE patients revealed gene signatures correlating with dsDNA antibodies production, low complement levels and nephritis, underscoring the essential role of T cells in the development of SLE [**25**].

To bridge the gap between non-coding GWAS discoveries and downstream affected genes, transcriptome-wide-association studies (TWAS) have received more attention over the last years. TWAS leverage predictive models of expression, through integrating GWAS findings and gene expression reference panels, in order to uncover gene-complex traits associations. To this end, Yin et al performed – for the first time – a TWAS for SLE, identifying 276 candidate genes and demonstrating the genetically regulated transcriptional activity of ACAP1 in the context of SLE [**26**].

Epigenetics

Epigenetic mechanisms of gene regulation including the DNA methylation, non-coding RNAs and the histone modifications, are thought to be closely related to the pathogenesis of SLE. Global DNA hypomethylation has been reported in T cells from patients with active SLE, resulting in heightened expression of autoimmune-related, methylation-sensitive genes, such as ITGAL, TNFSF7, CD40L IL6, IL10, IL13, CD6 and CD11A [**27**]. Additionally, Tsokos et al demonstrated that in SLE T cells, DNA methyltransferase 1 (DNMT1) and DNMT3a downregulation leads to hypomethylation of the PP2Acα promoter and subsequent enhanced binding of the transcriptional enhancer p-CREB, which is linked to overexpression of the SLE associated PP2Acα [**28**]. Accordingly, methylation intensity of the PP2Acα promoter displayed an inverse correlation with SLE activity [**28**]. Interestingly, genome-wide methylation studies in naïve CD4+ T cells from SLE patients revealed significant hypomethylation in several type I IFN-regulated gene loci, arguing for a potential pathogenetic implication of the aberrant DNA methylation in SLE [**29**, **30**].
Abnormal histone modification patterns have been reported in splenocytes from MRL/lpr lupus-prone mice [**31**]. Moreover, global histone H3 and H4 hypoacetylation have been observed in CD4+ T cells from patients with active SLE [**32**]. Taking a step forward, Hedrich et al showed evidence that in SLE T cells enhanced expression of the transcription factor cAMP-responsive element modulator (CREM)α facilitates the recruitment of the histone deacetylase 1 (HDAC1) to the IL2 promoter, contributing -through histone H3K18

deacetylation- to the transcriptional repression of the IL2 gene, a hallmark of the human SLE T cells [**33**]. On the other hand, increased CREMa binding within the IL17A promoter results in decreased recruitment of the HDAC1 and DNMT3a at this site, which in turn might account for heightened expression of IL17 by CD4+ T cells in SLE [**34**]. Of note, administration of the HDAC inhibitor suberoylanilide hydroxamic acid has shown encouraging results in lupus-prone mice, underscoring the potential role of the epigenetic modifications as therapeutic targets in SLE [**35**].

Complete understanding of the non-coding RNA regulation in SLE still remains elusive. Lashine et al suggested that the downregulation of the miR-155 in PBMCs from SLE patients might associated with decreased IL2 production through augmented expression of the protein phosphatase 2A (PP2A) [**36**]. Additionally, the miR-146a, which functions as a negative regulator of type I IFN pathway in PBMCs from SLE patients, has been found to inversely correlate with disease activity [**37**]. Several studies indicated the potential impact of crosstalk between epigenetic modifications on the transcription regulation in SLE. For example, miR-148a and miR-126, which are both overexpressed in SLE CD4+ T cells, might contribute to T cell autoreactivity via suppression of DNMT1 and subsequent DNA-hypomethylation [**38**].

Overview of the SLE pathogenesis

Aberrant clearance of apoptotic material, deregulated nucleic acid sensing, abnormal lymphocyte activation, signal transduction and cytokine production, as well as impaired degradation of neutrophil extracellular traps (NETs) are key concepts around the pathogenesis of SLE, leading to loss of tolerance and tissue damage. Accordingly, multiple subsets of immune cells display defective phenotypes and functions in the context of SLE (**Figure 4.**).

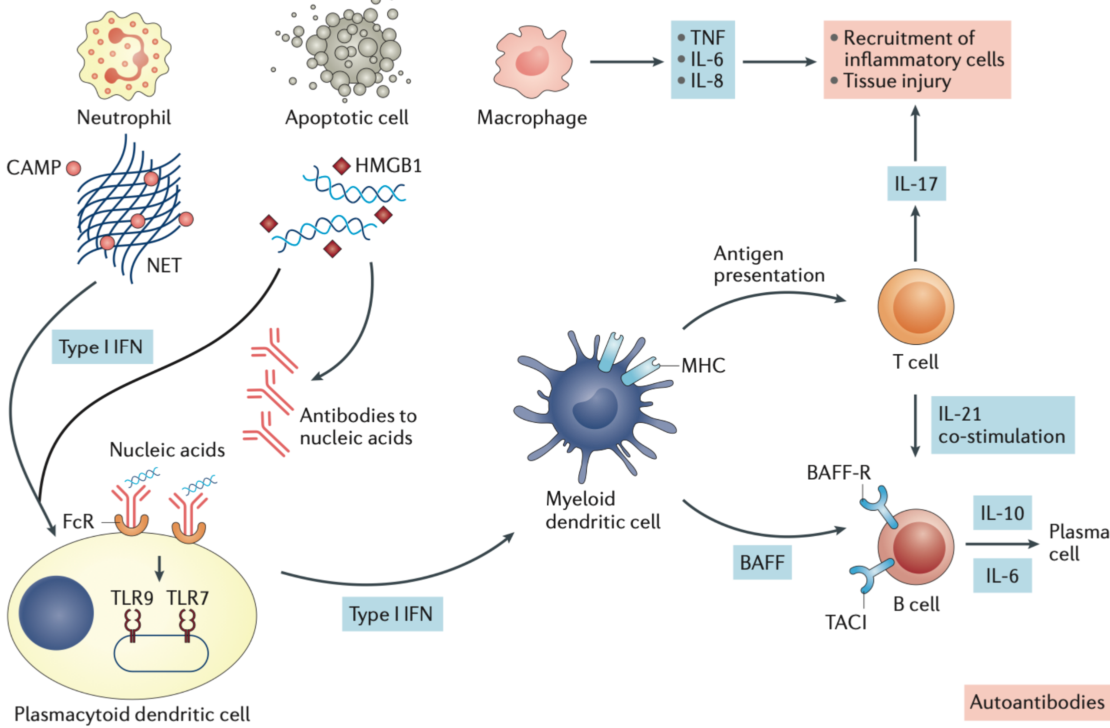

Figure 4. Overview of the SLE pathogenesis. BAFF, B-cell activating factor; BAFF-R, BAFF receptor; CAMP, cathelicidin antimicrobial peptide; FcR, Fc receptor; MHC, major histocompatibility complex; TACI, transmembrane activator and cyclophilin ligand interactor; TLR, Toll-like receptor. Adopted by Tsokos et al., 2021.

T cells

Loss of T cell tolerance is thought to play an indispensable role in the occurrence and the development of SLE. SLE human T cells are chronically activated and are characterized by aberrant signaling through the T-cell receptor (TCR) [**19**]. Specifically, in T cells from SLE patients, the expression levels of the CD3ζ chain are significantly downregulated and the TCR-CD3 complex frequently bears a substitution by the homologous Fc receptor common gamma subunit chain (FcRγ) [**19**]. Rather than the tyrosine-protein kinase ZAP-70, which pairs the CD3ζ chain, FcRγ recruits the tyrosine-protein kinase SYK, contributing to the hyperactivated phenotype of the T cells in SLE [**19**, **39**]. Notably, alterations of the expression and composition of the lipid rafts have been described in SLE T cells, promoting the excessive T cell activation [**19**].

Immunometabolism has emerged as central mechanism for the regulation of T cell responses. Chronic activation of autoreactive T cells in SLE results in persistent mitochondrial

hyperpolarization [**40**]. Indicative of the oxidative stress, which characterizes SLE T cells, is the depletion of the major intracellular antioxidant glutathione in the plasma of SLE patients as well as the beneficial effect of the N-acetylcysteine on disease activity in a pilot SLE clinical trial [**40**].

Although SLE T cells display a hyperactive phenotype, decreased production of IL2 by T cells represents a hallmark of SLE [**19**]. Besides its central role in the development and function of immunosuppressive T_{reg} cells, IL2 constrains IL17 production, which is abnormally elevated in serum from SLE patients [**40**]. Double-negative T cells (CD4−CD8−) were shown to produce increased amounts of IL17 in SLE [**40**]. Notably, double-negative T cells are expanded in SLE and infiltrate the kidneys of both patients and lupus-prone mice, amplifying local inflammation and tissue damage [**40**].

T cell-B cell interactions represent a crucial checkpoint in the process of secondary B cell maturation and the maintenance of tolerance. In SLE, T cell-B cell interactions are aberrant, often occurring in tertiary lymphoid organs and are more transient compared to healthy individuals [**19**, **40**]. T follicular helper (T_{fh}) cells constitute a subset of effector T cells, essential for B-cell maturation and immunoglobulin production. Specifically, T_{fh} cells produce IL21 and provide the necessary receptor engagement in the germinal center, facilitating isotype switching and somatic hypermutation. T_{fh} cells are expanded in spleens of MRL/lpr mice [**41**], whereas circulating T_{fh} cell are increased in SLE patients and were correlated with plasmablasts as well as the anti-ds DNA autoantibodies titers [**40**, **42**]. In addition, extrafollicular helper T cells (eT_{fh}) represent an anatomically distinct CD4+T cell subpopulation that regulate plasma cell differentiation outside the follicle [**44**]. Remarkably, Liarski et al suggested that T_{fh} cells are evident within lymphoid aggregates in renal biopsies from patients with active LN [**42**, **43**]. In this line, Yin et al showed evidence for abnormal renal accumulation of γδ2 T cells, an IL21-secreting subpopulation of γδ T cells, which might support the formation of extrafollicular germinal centers in SLE kidney [**45**].

Cytotoxic CD8+ T cells in SLE exhibit reduced cytolytic capacity, contributing to the increased risk of infections that defines the disease [**46**]. To this end, expansion of a dysfunctional CD38+CD8+ T cell subset, with features of reduced granzyme and perforin production was reported in peripheral blood from SLE patients [**46**]. Of note, emerging evidence suggests that in SLE, self-reactive CD8+ T cells tend to lose CD8 expression, turning into PD-1 expressing, double-negative T cells, which display impaired anti-viral responses *in vitro* [**47**]. In addition, in SLE patients, prolonged type I IFN exposure promotes CD8+ T cells apoptosis via metabolic rewiring [**48**].

B cells

Although SLE presents extraordinary heterogeneity, production of autoantibodies is nearly universal among patients with SLE. SLE is characterized by increased numbers of self-reactive B cells, both in new emigrant and mature naïve B cell compartments, indicating profound defects in B cell tolerance. Specifically, pronounced naive B cell (CD19+CD27+) lymphopenia, expansion of transitional B cell (CD19+CD24^{hi}CD38^{hi}), switched memory B cell (CD19+CD27+IgD+), double-negative (CD19+CD27+IgD+) B cell and plasmablast/plasma cell (CD27^{hi}CD38+CD19^{dim}sIg^{low}CD20+CD138+) populations are associated with active disease [**19**]. In addition, impaired regulatory capacity of the CD19+CD24^{hi}CD38^{hi} B cells, which suppress the differentiation of T helper 1 cells -partially via the secretion of IL10- have been described in SLE [**49**].

Several B cell intrinsic risk alleles are linked to loss of B cell tolerance, amplifying the loop of autoimmunity in SLE [**50**]. For example, in patients with SLE, risk alleles for BANK1, BLK, CSK, and FCGR2B might contribute to the increased B cell activation via hyper-responsiveness to B cell receptor (BCR) engagement, whereas PTPN22 risk allele might lead to hypomethylation of proteins included in BCR signaling pathway, resulting in dampening of tolerance in immature B cells [**50**].

Unlikely dendritic cells, in B cells, specific antigen uptake is mediated through BCR, which after engagement, bound to antigen undergoes endocytosis and proceeds via intracellular routing to TLR7- and TLR9-containing late endosomes, resulting in TLR-induced B cell co-stimulation [**51**]. In SLE, breach of B cell tolerance to autoantigens is at least partially regulated in a cell-intrinsic manner by TLRs [**51**]. To this end, B cell-specific TLR7 deletion prevents formation of autoantibodies against RNA-associated autoantigens and limits systemic autoimmunity in lupus-prone mice [**52**], whereas B cell-intrinsic TLR9 deletion restrained TLR7-mediated spontaneous autoimmunity in C57BL/6 mice [**53**]. In this line, selective B cell-inactivation of TLR signaling adaptor Myd88 ameliorates nephritis in MRL/lpr mice [**19**].

Aberrant cytokine production is strongly implicated with loss of B cell tolerance in SLE. B-cell activity factor (BAFF) is a critical survival factor for transitional and mature B cells and excess of BAFF rescues self-reactive B cells from peripheral deletion. Serum levels of BAFF are increased in patients with SLE and correlate with the presence of autoantibodies [**54**].

Moreover, BAFF overexpression in lupus prone mouse model leads to a striking acceleration of glomerular pathology [**55**]. Of note, B-cell depletion therapy in patients with SLE often results in elevated levels of BAFF, posing the concern that repopulating B cell subsets could exert an autoreactive phenotype [**56**]. To this end, targeted BAFF inhibition represented a reasonable therapeutic approach in SLE, leading to the approval of the anti-BAFF monoclonal antibody belimumab from the FDA for the therapy of patients with active SLE.

Neutrophils

Dysregulated functional properties of neutrophils have been reported in SLE. Impaired phagocytic capacity of SLE-derived neutrophils is well established, predisposing to infections [**19**]. Accordingly, Lupus Erythematosus (LE) Cell, a blood granulocyte that have engulfed opsonized apoptotic remnants, was the first neutrophil abnormality discovered in bone marrow of SLE patients. Moreover, diminished generation of reactive oxygen species (ROS) characterizes neutrophils from SLE patients and is associated with increased disease activity and organ damage [**57**]. Notably, reduced ROS production might affect the apoptopic pathway, promoting defective clearance of cell remnants and autoantigen exposure in SLE [**40**]. Although neutrophils in SLE exhibit decreased phagocytic activity, they display an activated phenotype and overexpress adhesion molecules [**19**].

NETosis is a regulated form of neutrophil cell death that contributes to host defense against pathogens and involves extrusion of chromatin decorated with proinflammatory cytokines and antimicrobial proteins. Of note, this extruded material serves as source of citrullinated peptide and nucleic acid antigens, driving autoantibody production in SLE [**40**]. Enhanced NET formation does appear to occur in SLE *in vivo* [**58**]. SLE is characterized by elevated levels of low-density granulocytes –a pathologic neutrophil subset-, which exhibit increased capacity to form neutrophil extracellular traps (NETs) [**40**]. Netting neutrophils are major amplifiers of type I IFN production in SLE [**58**]. Specifically, Garcia-Romo et al showed evidence that NETs containing DNA as well as large amounts of antimicrobial peptides induce type I IFN production by plasmacytoid dendritic cells (pDCs) in a TLR9-dependent manner [**58**]. In turn, enhanced type I IFN stimulates NET formation in SLE, indicating the presence of a positive feedback loop [**40**].

Dendritic cells

Dendritic cells (DC) are professional phagocytes, implicated in clearance of apoptotic material and presentation of self-antigens, thus serving as critical link between innate and adaptive immune system. Patients with SLE present decreased number of circulating conventional DC, but expansion of the pDCs subpopulation [**40**]. Excessive accumulation of pDCs has been described in kidney biopsies from SLE patients and cutaneous lupus lesions, implying their contribution to local tissue damage [19]. pDCs have been proposed as a major source of type I IFN in SLE [**59**]. Specifically, immunocomplexes containing self-nucleic acids activate DCs, resulting in type I IFN secretion via TLR7 and TLR9 stimulation [**40**]. In addition to its cytotoxic effects on variety of cells, which might facilitate increased autoantigen exposure, type I IFN directly affects T cells, promoting their survival, activation, and proliferation [**40**]. Moreover, type I IFN sensing by B cells decreases threshold for BCR stimulation, modulates antigen presentation, survival, and cytokine production, and promotes alterations in B cell development process, including arrested development at the early stages and expansion of B cells at transitional stage [**60**].

Conventional DCs are essential for antigen presentation, priming naïve T cells upon antigen uptake and maturation induced by appropriate maturation signals. To this end, RNA sensing by conventional DCs has been demonstrated to play a principal role in driving LN in conditional SLE mice overexpressing TLR7 [**61**]. Notably, DCs are major providers of BAFF, promoting survival and activation of autoreactive B cells in SLE.

Monocytes/Macrophages

Monocytes and macrophages represent an essential arm of innate immunity exhibiting versatile immunoregulatory, inflammatory and tissue repairing capabilities and thus playing an instrumental role in the development of SLE [**62**]. Macrophage depletion ameliorates nephritis mediated by pathogenic antibodies in lupus prone mice [**63**]. Along this line, renal macrophage infiltration represents a strong prognostic factor towards development of proliferative LN [**40**]. Aberrations in monocyte/macrophage-mediated CD40/CD40L co-stimulation contribute to the polyclonal B cell hyperactivity, which defines SLE [**62**]. SLE patients demonstrated a significantly higher number of circulating CD40L-expressing macrophages compared to healthy individuals [**62**]. Taking a step forward, data from murine

studies showed that CD40L overexpression by B cells induces SLE like phenotype, while anti-CD40L treatment prevents activation of self-reactive B cells as well as generation of autoantibodies in lupus mouse models [**62**, **64**]. Notably, elevated IFNa levels in serum of SLE patients induce differentiation of monocytes into DCs, promoting self-antigen presentation to autoreactive T and B cells [**62**].

Monocyte-macrophage lineage cells from patients with active SLE consistently overexpress adhesion molecules, which are essential for cell migration [**62**]. Accordingly, increased monocyte recruitment into blood vessels might contribute to the accelerated atherosclerosis process, which defines SLE [**40**].

Impaired clearance of apoptotic material serves as an important trigger of autoimmunity in SLE. Non-inflammatory phagocytosis of apoptotic cells by monocyte-derived macrophages obtained from SLE patients is impaired, resulting in increased accumulation of nuclear autoantigens in the germinal centers of the lymph nodes [**65**]. In addition, defective reticuloendothelial system Fc-receptor function accounts for the prolonged circulation of the immune complexes in SLE [**66**]. In this line, Kavai et al demonstrated that decreased Fc receptor expression and function on macrophages associated with active disease and renal involvement in SLE [**67**].

Unbalanced macrophage polarization towards M1 phenotype has been implicated with the SLE pathogenesis [**62**]. Macrophages from patients display excessive production of proinflammatory cytokines, including interleukin (IL)1β, IL6, tumor necrosis factor alpha (TNFa), IFNγ and C-C motif chemokine ligand 2 (CCL2) [**62**]. Additionally, SLE monocytes secrete large amounts of BAFF, a crucial cytokine for the survival of autoreactive B cells [**62**]. Despite M1 predominance, enhanced production of IL10, which directs macrophage polarization to an immunosuppressive phenotype, has been reported in SLE. Monocytes are an important source of IL10 in the peripheral blood of SLE patients, while priming with IFNa unleashes the proinflammatory functions of IL10, including induction of antibody production [**68**, **69**].

Unmet medical needs in SLE – Aim of the study

SLE is a disease of complex etiology, characterized by the failure of multiple regulatory mechanisms within the immune network. Despite the advances in understanding of pathogenesis of SLE, there are still important unmet medical needs in the management of SLE patients. Among others, the late diagnosis, the largely unpredictable disease course, the

lack of effective biomarkers, the increased morbidity and mortality, the damage accrual, the co-morbidities, the residual disease activity, the frequent flares, and the toxicity of the majority of the treatments remain substantial burdens for patients with SLE. The great challenges posed by the vastly diverse nature of SLE, and the paucity of informative outcome measures are reflected into the largely modest results of many SLE clinical trials. Over the last decades, only belimumab, anifrolumab and voclosporin demonstrated efficacy in randomized controlled clinical trials and received FDA approval for treatment of patients with SLE, underscoring the urgent need for novel therapeutic agents in the disease.

It is important to note, that current classification criteria and disease activity assessment tools do not necessarily capture the entire range of pathophysiological processes underlying SLE. To this end, several high-throughput strategies have proposed SLE subtypes as distinct disease entities based on molecular portraits. For example, Toro-Domínguez et al developed the scoring system MyPROSLE (Molecular dYsregulated PROfiles of SLE patients), which enabled the stratification of SLE patients based on immune related gene-modules and successfully predicted different clinical outcomes [**70**]. Similarly, Banchereau et al employed a personalized transcriptional immunomonitoring approach, which facilitated the classification of the SLE patients based on the immune networks best correlating with disease activity in each patient [**23**].

Herein, we sought to establish a computational pipeline, which could facilitate the stratification of the SLE patients according to their whole blood transcriptional profiles, irrespective of their clinical annotation. Taking a step forward, we used our molecular taxonomy approach to optimize therapeutic decisions in a personalized medicine approach. Lastly, we proposed novel compounds that could counteract the transcriptional aberrations of SLE patients in a targeted manner.

Molecular Taxonomy of Systemic Lupus Erythematosus Through Data-driven Patient Stratification: Molecular Endotypes and Cluster-tailored Drugs

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Molecular Taxonomy of Systemic Lupus Erythematosus Through Data-Driven Patient Stratification: Molecular Endotypes and Cluster-Tailored Drugs

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Objectives: Treatment of Systemic Lupus Erythematosus (SLE) is characterized by a largely empirical approach and relative paucity of novel compound development. We sought to stratify SLE patients based on their molecular phenotype and identify putative therapeutic compounds for each molecular fingerprint.

Methods: By the use of whole blood RNA-seq data from 120 SLE patients, and in a data-driven, clinically unbiased manner, we established modules of commonly regulated genes (molecular endotypes) and re-stratified patients through hierarchical clustering. Disease activity and severity were assessed using SLEDAI-2K and Lupus Severity Index, respectively. Through an *in silico* drug prediction pipeline, we investigated drugs currently in use, tested in lupus clinical trials, and listed in the iLINCS prediction databases, for their ability to reverse the gene expression signatures in each molecular endotype. Drug repurposing analysis was also performed to identify perturbagens that counteract group-specific SLE signatures.

Results: Molecular taxonomy identified five lupus endotypes, each characterized by a unique gene module enrichment pattern. Neutrophilic signature group consisted primarily of patients with active lupus nephritis, while the B-cell expression group included patients with constitutional features. Patients with moderate severity and serologic activity

exhibited a signature enriched for metabolic processes. Mild disease was distributed in two groups, exhibiting enhanced basic cellular functions, myelopoiesis, and autophagy. Bortezomib was predicted to reverse disturbances in the "neutrophilic" cluster, azathioprine and ixazomib in the "B-cell" cluster, and fostamatinib in the "metabolic" patient subgroup.

Conclusion: The clinical spectrum of SLE encompasses distinct molecular endotypes, each defined by unique pathophysiologic aberrancies potentially reversible by distinct compounds.

Keywords: molecular taxonomy, drug response prediction, systemic lupus erythematosus, drug repurposing, endotypes

INTRODUCTION

Systemic lupus erythematosus (SLE) has a unique set of attributes, which has established it as the prototype among systemic autoimmune diseases. With few notable exceptions, recent advances in the understanding of SLE pathogenesis have failed to translate into new therapies. High-throughput methods have enabled the discovery of novel drugs in a time- and cost-efficient manner. To this end, the Connectivity Map (CMap) project is the first powerful drug repurposing platform that embedded gene expression responses of 4 human cell lines treated with different doses of a large collection of FDA-approved compounds (1). Taking a step forward, the NIH-supported Library of Integrated Network-Based Cellular Signatures (LINCS) enriched the transcriptomic databases of the CMap project by integrating the gene expression profiles of more than 60 cell lines before and after exposure to more than 20,000 perturbagens (2). In this context, Toro-Dominguez et al. employed the successor of the CMap, Lincscloud, suggesting the therapeutic potential of phosphoinositol 3 kinase and mammalian target of rapamycin (mTOR) inhibitors in SLE (3).

We have previously used mRNA sequencing to define the transcriptomic signature of SLE patients. Our data showed that SLE is characterized by a "susceptibility signature" present in patients in clinical remission compared to healthy controls. Additionally, we identified an "activity signature" present in patients with active disease, which was mainly associated with genes that regulate immune cell metabolism, protein synthesis and proliferation. Lastly, we detected a "severity signature", best illustrated in active nephritis, linked to granulocyte and plasmablast/plasma–cell pathways (4).

In the present study, we used the same RNA-sequencing dataset in order to stratify lupus patients according to underlying fundamental molecular aberrancies and predict personalized therapeutic options. Specifically, we established an *in silico* drug prediction pipeline to select the optimal treatments for each patient subgroup, among compounds that have already been tested against SLE in clinical trials. We also deployed a personalized drug repurposing pipeline to identify FDA-approved drugs or patented compounds for different indications, that could be applied as potential therapeutic agents for each group of SLE patients. We provide a comprehensive, in-depth analysis of the human SLE transcriptome to guide precision care and new therapeutic compound development.

MATERIALS AND METHODS

Patients

Whole blood transcriptional profiles of 120 patients with SLE and 58 healthy individuals (4) were analyzed. Disease activity at the time of blood sampling was assessed by the modified Systemic Lupus Erythematosus Disease Activity Index 2000 (SLEDAI-2K), after exclusion of the serologic features (anti-dsDNA and complement levels) (clinical SLEDAI) (5). Remission was defined as a clinical SLEDAI-2K = 0 and daily prednisolone dose of ≤5 mg (6, 7). Active disease was defined as a clinical SLEDAI-2K ≥4. Irreversible organ damage was assessed using the SLICC damage index (SDI) (8). Lupus Severity Index was calculated for each patient (9).

Co-Expression Network Analysis

We employed CoCena² (construction of co-expression network analysis-automated, https://github.com/UlasThomas/CoCena2), using the 10,000 most variable genes as input, to determine modules of co-expressed transcripts. Next, agglomerative hierarchical clustering of patients, based on their group fold changes (GFC) for each cluster of co-expressed genes, defined the disease molecular endotypes. Functional enrichment analysis was performed using clusterProfilerR package (10).

Drug Prediction Analysis

DEseq2 was used to identify differentially expressed genes (DEGs) specific for each patient's endotype (11). We obtained gene expression signatures of drugs that are incorporated in the treatment recommendations for SLE (12), or have failed to reach SLE clinical trials endpoints and are included in the following iLINCS sublibraries: i) iLINCS chemical perturbagens (LINCSCP); ii) iLINCS targeted proteomics signatures (LINCSTP); iii) Disease-related signatures (GDS); iv) Connectivity Map signatures (CMAP); v) DrugMatrix signatures (DM); vi) Transcriptional signatures from EBI Expression Atlas (EBI); vii) Cancer therapeutics response signatures (CTRS); and viii) Pharmacogenomics transcriptional

signatures (PG). These were downloaded using the iLINCS API (https://github.com/ucbd2k/ilincsAPI/blob/master/usingIlincsApis.Rmd). Statistically significant DEGs from each drug signature were ordered by decreasing fold change magnitude. The top 300 DEGs were selected and upregulated/downregulated genes were identified. Gene set enrichment analysis (GSEA) was performed using fgsea R package (13). To determine the optimal number of drug clusters for k-means clustering, the elbow method was applied.

Drug Repurposing Analysis

Drug repurposing results were prioritized using the bioinformatic tool CoDReS (Computational Drug repositioning score) (14), which enables the exploration of compound drugability, based on an algorithm that combines functional and structural scores. The functional score quantifies the pharmacodynamic potential of a compound by assessing its association to SLE hallmarks. This potential includes the binding affinity to SLE molecular targets (enzyme, receptor, transcription factor, etc.), as well as the overlap of its genomic targets with genes implicated in the pathogenesis of the disease. The structural score pertains to the pharmacokinetic properties of compounds and contains information related to the hydrophilic-lipophilic balance, solubility, permeability, as well as oral bioavailability of a drug candidate, based on the "Lipinski rules of 5" (15) and "Veber's rule" (16).

RESULTS

Co-Expression Analysis Stratifies SLE Patients Into Distinct Endotypes in an Unbiased Data-Driven Manner

Applying the CoCena² pipeline, we identified nine modules of co-expressed transcripts illustrated with different colors in **Figure S1**. Hierarchical clustering of samples according to each module's group fold changes (GFC) reassigned patients into five groups (G1 to G5) (**Figures 1A, B**). To define disease-driving molecular mechanisms, we investigated the CoCena²-derived modules enrichment in each patient group (**Figures 1C, D**). Interestingly, groups displayed distinct enrichment patterns, each exhibiting unique major module predominance. Platelet activation and hemostasis were identified as two group 1 specific signals (G1, "Hemostasis" group), overrepresented in the *orchid module*. Detailed functional enrichment analysis of the *dark-grey module* revealed that autophagy-associated signatures were prominently enriched in patient group 2 (G2, "Autophagy" group). Macroautophagy disturbances in G2 are accompanied by deregulation of pathways involved in neutrophil activation and toll-like receptor (TLR) cascade. Combined enrichment of the *pink module*, linked to aberrancies of mRNA splicing and mRNA surveillance mechanisms, and the *dark-orange module*, implicated among others in mitochondrial dysfunction, efficiently distinguished group 3 (G3, "Metabolism" group). Heightened expression of the *indian-red module*, which predominantly consists of genes implicated in neutrophil activation and degranulation, defines group 4 (G4, "Neutrophil" group). Enrichment of the *dark-green module*, which comprises genes (such as CD38, BLNK, IGHA1, TNFRSF17, CD22, CD79A, MS4A1, IGHD) linked to B-cell and plasmablast-mediated responses, was indicative of group 5 (G5, "B cell" group). Interestingly, G5 displays a concurrent increased expression of the steel-blue module, which is associated with type I interferon (IFN) signaling.

Molecular Clusters Are Associated With Distinct Clinical Traits

To evaluate the clinical implications of molecular endotype characterization, we next assessed each group's clinical features, including demographics, clinical manifestations, serologic features, and administered treatments. The "Neutrophil" group (G4, n= 11, 9.1% of the total cohort) almost uniformly encompassed patients with active lupus nephritis (n=9/11) (**Figure 2**). Patients of this cluster also exhibited high serologic and clinical activity; the majority were treated with cyclophosphamide at the time of blood sampling (**Figures 2**, **S2**, **S3**). The "B-cell" group (G5, n=18, 15% of the total cohort) was characterized by high prevalence of constitutional symptoms. Although statistical significance was not reached, a tendency to a higher frequency of hematological and neurological manifestations was apparent in this cluster. Mucocutaneous and musculoskeletal manifestations were most common in the "Metabolism" group (G3, n=30, 25% of the total cohort), occurring in 63% and 50% of patients, respectively, while a history of neuropsychiatric SLE (NPSLE) was reported in 27%. Interestingly, the clinically heterogenous "Hemostasis" group (G1, 24.2% of the total cohort) was characterized by high frequency of male patients, while Disease Modifying Anti-Rheumatic Drugs (DMARDs) were the most commonly used therapy. Finally, the "Autophagy" group (G2, n=32, 26.7% of the total cohort) consisted of patients with mild to moderate SLE. Accordingly, photosensitivity and malar rash were found in 59,3% and 81,2% of the patients of G2, respectively.

Molecular Endotypes Can Be Used to Predict Group-Specific Effective Compounds Towards Personalized Therapeutic Decisions

To explore personalized therapeutic solutions, we identified compounds tailored to each group's molecular fingerprint. This was achieved through leveraging our CoCena² based co-expression analysis, to establish an *in silico*, signature-based, drug prediction pipeline. As group-specific signatures, we employed the DEGs resulting from the comparison of each SLE endotype with a pool of 58 healthy controls.

To this end, we initially collected the transcriptional profiles corresponding to cellular responses against drugs that are either currently used in clinical practice, are or have failed in SLE clinical trials and are listed in the iLINCS prediction databases (**Table S1**). Our query returned 3,900 drug signatures (**Table S2**). Using SLE group-specific transcriptional profiles as input, we performed GSEA against the datasets of the top upregulated and

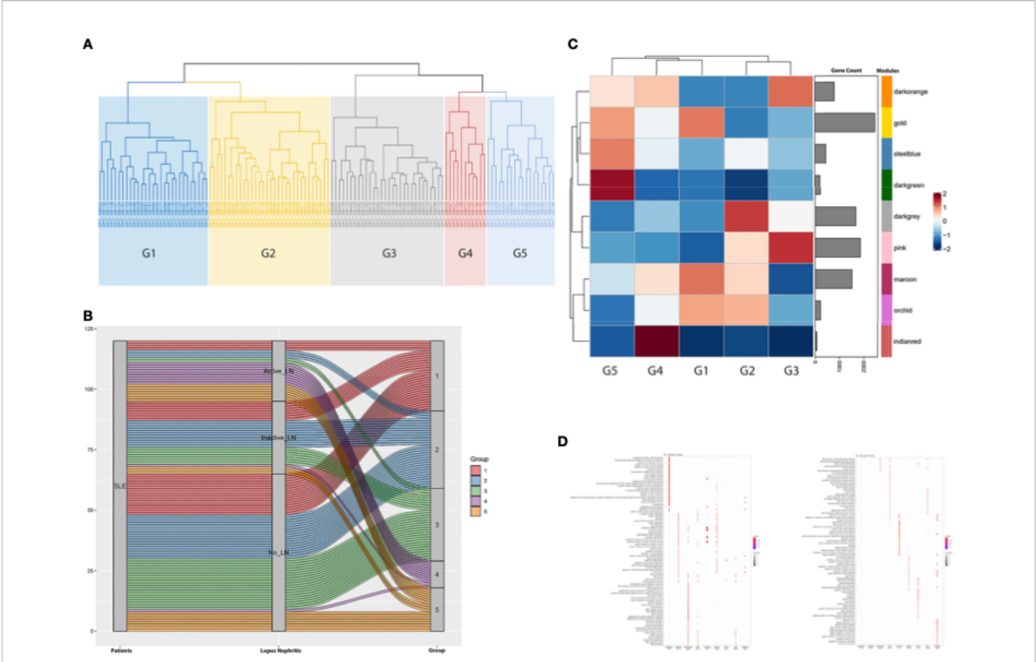

FIGURE 1 | (A) Hierarchical clustering of the samples based on the magnitude of the expression of each gene module (identified in **Figure S1**) defined five groups of patients (G1 to G5). Briefly, the x-axis demonstrates the patients analyzed in our study. GFC denotes the Group Fold Changes (defined in **Figure S1**); GB denotes the sample; the number after the GB acronym denotes each patient database ID. **(B)** Alluvium plot illustrating the distribution of the SLE patients into the patient groups G1-G5 generated after the hierarchical clustering of the samples according to each module's group fold changes. Briefly, the 120 SLE patients included in our study are displayed in the left vertical box (Patients). Each horizontal block corresponds to a patient. The distribution of the patients according to the presence and the activity of Lupus Nephritis (LN) was demonstrated in the middle vertical box. The distribution of the patients into the five CoCena2 analysis defined patient groups was shown in the right vertical box. **(C)** Heatmap showing the mean of the GFCs of the CoCena2 analysis derived gene modules in each one of the previously defined patient groups. Group specific GFCs demonstrated similar and counteracting gene expression patterns among patient groups. Briefly, increased expression of the indian-red module characterized G4. Enrichment of the dark-green module defined G5. Heightened expression of the dark-grey module distinguished G2. Lastly, enrichment of the pink and dark-orange modules was indicative of G3. **(D)** Dot plot displaying the functional enrichment analysis of the CoCena2-derived modules. Gene modules are shown on the basis of the graph. Enriched gene ontologies and pathways are shown on left side of the graph. Briefly, the indian-red module included genes that were mainly enriched in neutrophil activation and degranulation. Functional enrichment analysis of the dark-green module revealed disturbances related to plasmablast-mediated responses. Dark-grey module predominantly consisted of genes related to autophagy. Genes of the pink module were enriched in mRNA splicing, whereas gene ontologies related to mitochondrial function were overrepresented among the genes included in the dark-orange module.

top downregulated DEGs for each drug signature and normalized enrichment scores (NES) were defined. Next, we calculated the difference (ΔNES) of the NES from the downregulated gene set and the NES from the upregulated gene set for each drug signature per SLE cluster (**Table S3**). Accordingly, a positive ΔNES indicated compounds that were predicted to reverse the group-specific transcriptomic aberrancies. To determine endotype-specific drug candidates, we applied k-means clustering, in order to group drug signatures according to ΔNES (**Figures S4**, **S5**). Drug signatures with the highest ΔNES within each drug cluster induce cellular transcriptional alterations which most efficiently counteract group-specific SLE signatures.

In G5, the top signatures were linked to azathioprine (ΔNES=2.76) and ixazomib (ΔNES=2.67) (**Figure 3A**), whereas in G2 to the proteasome inhibitor bortezomib (ΔNES=2.84). Signatures related to the SYK kinase inhibitor tamatinib (ΔNES=2.81) were top ranked in G3 subgroup (**Figure 3B**). In G4 group, signatures related to bortezomib occurred in high frequency (76%) among the top 50 signatures, starting with a ΔNES score 2.54 and, together with the calcineurin inhibitor cyclosporine (ΔNES score 2.49), might represent alternative G4-specific therapeutic options (**Figure 3C**). Finally, in both groups 4 and 5, signatures related to vitamin D derivatives (such as seacalcitol) prevailed, with a ΔNES score 3.04 and 2.97, respectively.

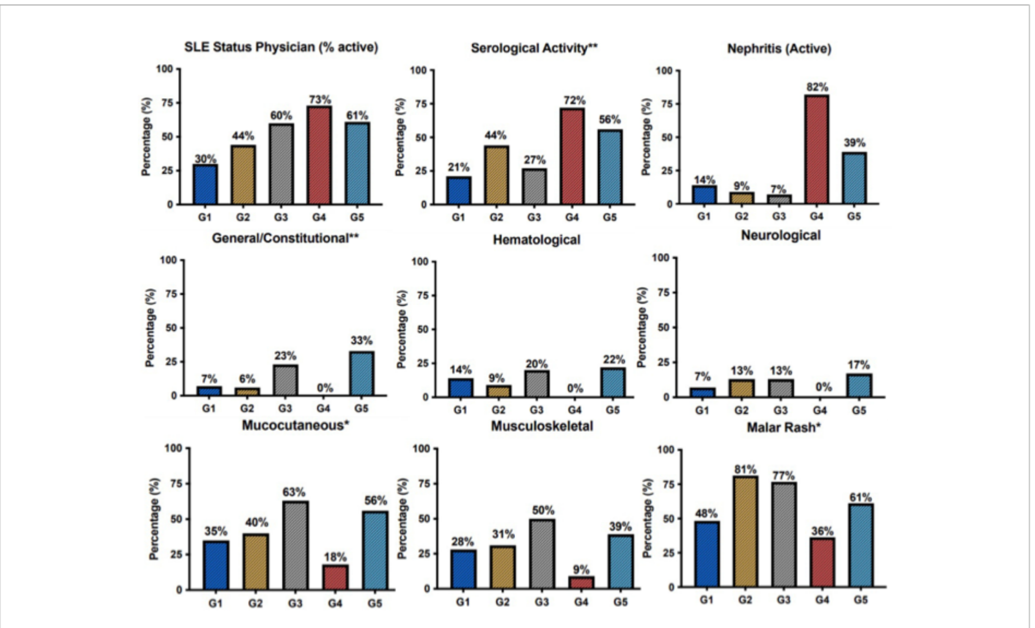

FIGURE 2 | Barplots demonstrating the prevalence of clinical features, Physician Global Assessment (SLE.status.(Physician)) and serological activity across patient groups. The G4 was defined by the high prevalence of active lupus nephritis. Constitutional symptoms occurred most frequently in the G5. Mucocutaneous and musculoskeletal manifestations were more prevalent among patients of the G3. $^{*}p < 0.05$; $^{**}p < 0.01$ in Kruskal-Wallis test, Chi-squared test.

Since the majority of the G4 patients were treated with cyclophosphamide at sampling, an agent that could drastically alter the whole blood transcriptional landscape, we divided G4 into two subgroups; one treated with cyclophosphamide (G4A, n=6/11) and a "cyclophosphamide-free" subgroup (G4B, n=5/11) and we applied the drug prediction pipeline. In accordance with our initial findings, bortezomib was overrepresented among the top 10 signatures in both subgroups (**Tables S4**, **S5**).

Drug Repurposing Tailored to SLE Molecular Aberrancies

Finally, we sought to propose new SLE therapeutic agents. To this end, we used a drug repurposing pipeline identifying patented compounds with potentially unrecognized efficacy in SLE. Using the iLINCS and CLUE platforms, we identified novel compounds that could reverse the previously defined SLE group-specific signatures. To sort out the top perturbagens derived from the iLINCS platform, we applied a negative concordance score cut-off of ≤ -0.5. Regarding the CLUE based analysis, only compounds exhibiting an inhibitory score of ≤ -50 were selected. Lastly, group-specific perturbagens were determined, as shown in the Venn diagram (**Figures 4A, B**). To enhance the performance of our approach, group-specific compounds were ranked, according to their druggability ("druggability prediction"). For this purpose, we used the bioinformatic tool CoDReS (Computational Drug Repositioning Score) (14). Uploading the iLINCS- and CLUE-derived compound lists (which were related exclusively to each SLE endotype) to the CoDReS platform resulted in the re-ranking of the repurposed drugs, according to their biological and pharmaceutical potential (**Tables S6–S15**).

G1 Subgroup

Our analysis indicated the p38 MAP kinase inhibitor vx-102 (17) and the TBK1 and IKK kinase inhibitor amlexanox, as potentially beneficial compounds. Lenalinomide, which has been tested in SLE clinical trials (18), and the c-met-HGFR (hepatocyte growth factor receptor) inhibitor pf-04217903 (19) might also be considered as treatment options for G1 SLE patients.

G2 Subgroup

The GSK3B/CDK double kinase inhibitor kenpaullone (20) was found to reverse G2-specific transcriptional patterns. Notably, the antidiabetic DPP4 inhibitor saxagliptin (21), the DNA methylation inhibitor zebularine [used for the treatment of CD4+ T cells mediated uveitis in a murine model (22)], the smoothened receptor antagonist erismodegib [inhibitor of the sonic hedgehog signaling (23)] were also identified as potential therapeutic compounds.

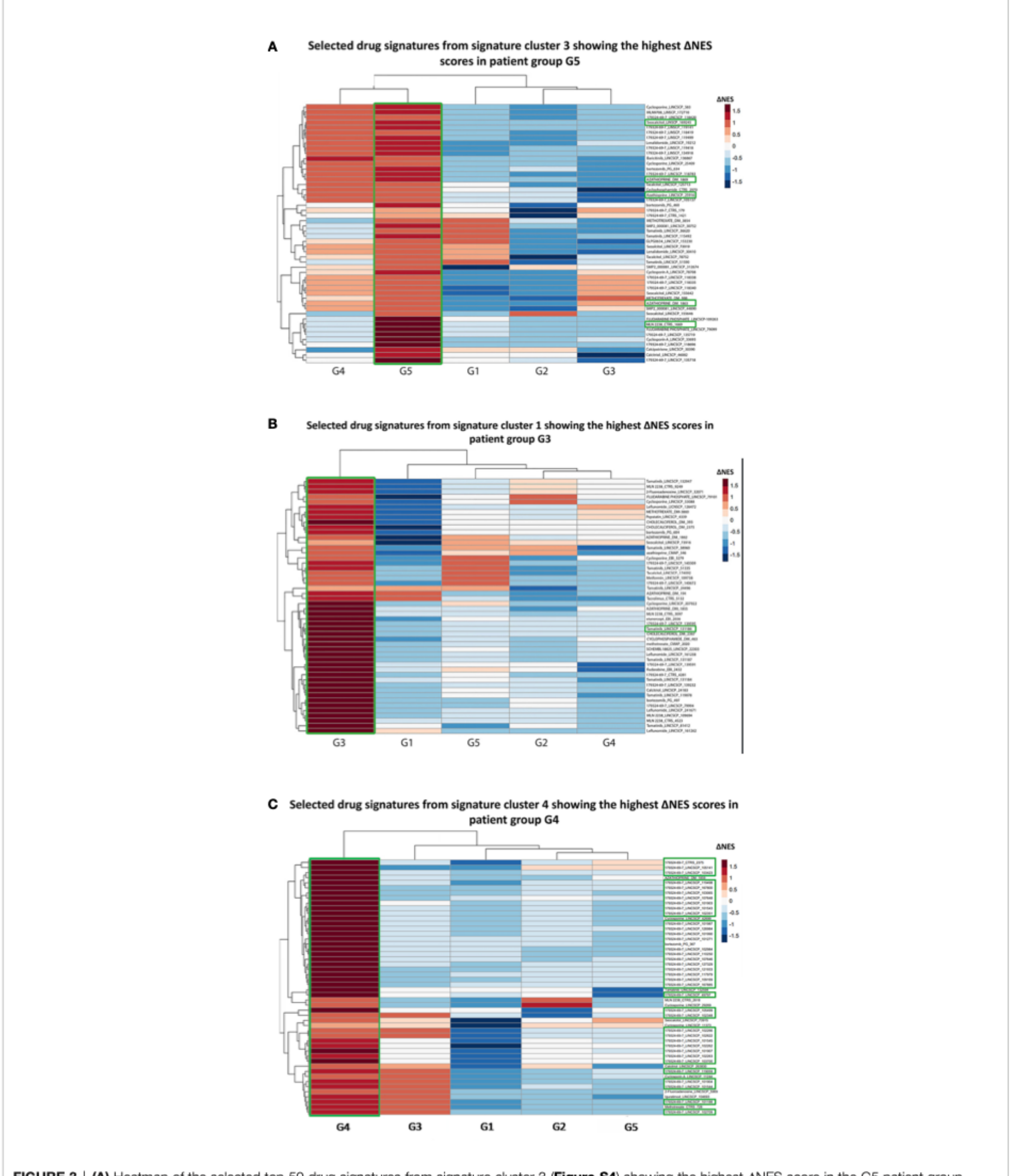

FIGURE 3 | **(A)** Heatmap of the selected top 50 drug signatures from signature cluster 3 (**Figure S4**) showing the highest ΔNES score in the G5 patient group. Signatures of the azathioprin and the ixazomib showed the highest ΔNES scores in the G5 patient group. MLN2238: Ixazomib. Labeling was carried out based on the following strategy: "drug name"_"database". **(B)** Heatmap of the selected top 50 drug signatures from signature cluster 1 with the highest ΔNES score in the G3 patient group. Signatures of SYK kinase inhibitor tamatinib showed the highest ΔNES scores in the G3 patient group. **(C)** Heatmap of the selected top 50 drug signatures from signature cluster 4 with the highest ΔNES score in the G4 patient group. 76% of the top 50 drug signatures for G4 patient group belonged to the proteasome inhibitor bortezomib. 179324-69-7: Bortezomib.

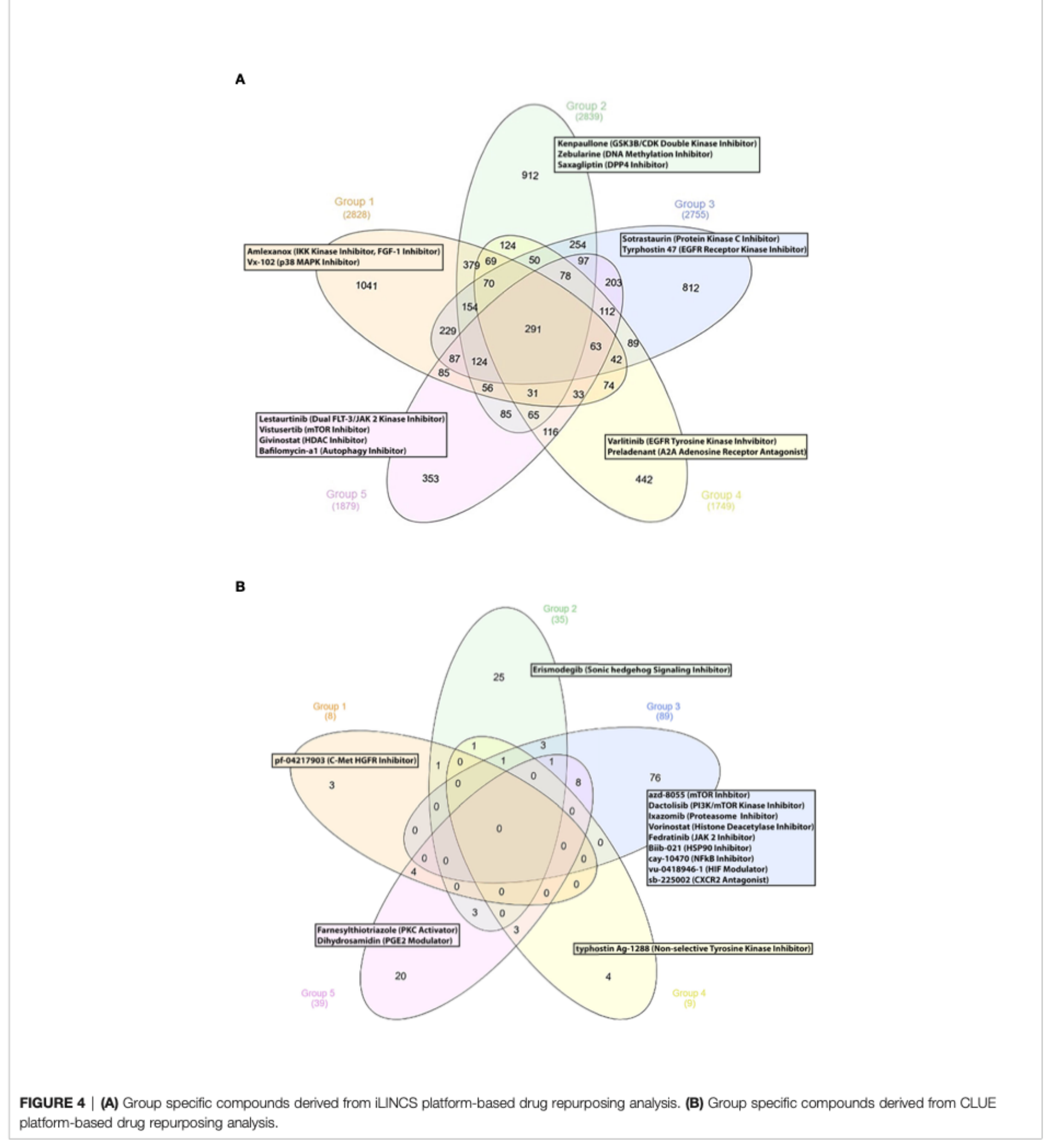

FIGURE 4 | **(A)** Group specific compounds derived from iLINCS platform-based drug repurposing analysis. **(B)** Group specific compounds derived from CLUE platform-based drug repurposing analysis.

Other Subgroups

Concerning G3, we identified numerous potential drug candidates, including the protein kinase c (PKC) inhibitor sotrastaurin (24), the EGFR receptor kinase inhibitor tyrphostin 47 (25), and the mTOR kinase inhibitors azd-8055, wye-125132, ku-0063794, wye-354 and torin-1 (26). Our data also underlined the potential role of the dual PI3K/mTOR kinase inhibitor dactolisib, the proteasome inhibitors ixazomib and mg-132 (27), the histone deacetylase inhibitors (HDACs) panobinostat, vorinostat, dacinostat, apicidin and merck60 (28, 29), and the HSP90 inhibitor biib021 (30) for potential treatment of patients in G3. Based on their pathophysiological relevance, the HIF (hypoxia inducible factor) modulator vu-0418946-1 (31), the NFkB inhibitor cay-10470 (32), the CXCR2

antagonist sb-225002 (33), the JAK2 inhibitor fedratinib (34), might represent promising therapeutic choices for G3 patients.

Moreover, chemical substances, such as the EGFR 2 receptor tyrosine kinase inhibitor varlitinib (25), the A2A adenosine receptor antagonist preladenant (35) and the niacin (vitamin B3) (36) were found to be G4-specific drug candidates.

Finally, small molecules, such as the artemisinin derivative artesunate, a drug applied for malaria (37), the dual FLT-3/JAK 2 kinase inhibitor lestaurtinib (38), the class1/2 HDAC inhibitor givinostat (39), the mTOR kinase inhibitor vistusertib (26) and the autophagy inhibitor bafilomycin-a1 (40) were identified as G5-specific compounds.

DISCUSSION

Despite advances in our understanding of SLE pathogenesis, selecting the optimal treatment for each individual patient remains a challenge. Herein, we applied a whole blood transcriptome-based molecular taxonomy strategy to stratify SLE patients according to their molecular fingerprints. Leveraging high-throughput computational methods, we exploited patient molecular endotypes to optimize putative therapeutic choices in a personalized approach. Finally, we applied available bioinformatic tools to establish a personalized drug repurposing methodology for the identification of new compounds that could enrich our armamentarium in SLE treatment.

Our data-driven re-stratification approach recapitulated the spectrum of previously identified lupus pathophysiological processes. For example, Banchereau et al. have shown that progression to active lupus nephritis is accompanied by an incremental enrichment of neutrophilic gene expression signatures (41). Accordingly, transcriptional signatures reflective of neutrophil activation defined G4 subgroup in our study, which consisted almost exclusively of active lupus nephritis patients.

Previous studies have highlighted the crucial role of type I IFN signaling in the loss of B cell tolerance and autoantibody production in SLE-prone mice (42). Gene expression signatures indicative of type I IFN production, B cells and plasmablast activation prevail in G5 group, implying the presence of type I IFN-induced autoreactive B cell development.

Incomplete response to existing drugs remains a substantial challenge for SLE patients, while various reasons related both to the disease and to trial design have accounted for the failure of several SLE clinical trials. Exploiting one of the largest drug signature databases to date, iLINCS, allowed us to predict the best patient endotype-specific drug candidates from a pool of currently available therapies and drugs. To this end, Alexander et al. have proposed the proteasome inhibitor bortezomib as a putative therapeutic option for patients with refractory lupus (43). Our unbiased approach indicated that use of bortezomib might be efficacious for the treatment of patients belonging to the "Neutrophil" molecular endotype. Moreover, expression of Syk is increased in SLE T cells and skin lesions of lupus MRL/lpr mice (44, 45), while administration of Syk inhibitors ameliorates kidney injury in lupus-prone mice (44). In this regard, our results suggest that patients in the G3 "Metabolism" subgroup might benefit most from treatment with fostamatinib. Depletion of abnormal plasma cells is considered a potential mechanism of action of the proteasome inhibitor ixazomib (46). In this context, our drug prediction analysis further substantiates the therapeutic relevance of targeting B cell responses in patients' group G5 ("B-cell" subgroup).

Over the last years, *in silico* drug repositioning studies for SLE have been published, based on gene expression and genetic profiles (47–49). Furthermore, efforts have been made to individualize drug repurposing results, according to the molecular features of lupus patients (49), whereas several studies have applied literature mining approaches, in order to prioritize the most promising compounds (50, 51). Herein, we performed personalized drug repurposing analysis using two robust, high-throughput platforms (iLINCS and CLUE). Notably, the top-ranked compounds were assessed not only through extensive literature review, but also according to their "druggability" profile. Activation of PI3K/Akt/mTORC1 signaling pathway characterizes T cells of SLE patients (52). In addition, pharmacological dampening of PI3K signaling in lupus-prone mice provides evidence for the therapeutic potential of targeting PI3K/Akt/mTORC1 pathway in SLE (52). Similarly, our findings indicate that several inhibitors of the PI3K/mTOR pathway (azd-8055, dactolisib) might be promising therapeutic options for patients belonging to the "Metabolism" (G3) group. Aberrant type I IFN and IFN-γ signaling and the encouraging results from baricitinib phase 2 study in SLE provide a clear rationale for targeting the JAK/STAT pathway in SLE (53). To this end, administration of the JAK2 inhibitor fedratinib, identified by our approach as an appropriate treatment for patients in G3 group, might also confer therapeutic benefit.

Certain limitations of our study deserve acknowledgment. First, the vast majority of patients included in this study were receiving immunosuppressive treatment at sampling, thus therapy-induced immunosuppression may be mirrored in the whole blood transcriptional profile, altering the expression of essential pathophysiological mechanisms. Moreover, our *in silico* drug prediction strategy is an explorative approach and additional *in vitro* and *in vivo* studies are clearly required to confirm our findings. Results of the phase III clinical trials BLISS-LN (54) and AURORA 1 (55) have shown a clinical benefit of adding belimumab or voclosporin, respectively, on top of standard-of-care in patients with lupus nephritis. Regarding the molecular complexity of the disease, also underscored by our findings, these studies might denote the need towards combination treatment approaches. Obviously, further drug combination prediction analysis might be useful to explore new avenues for SLE treatment.

In summary, we present a molecular taxonomy-based pipeline to guide therapy and identify new compounds for patients with SLE, based on a comprehensive, in-depth analysis of the transcriptome. These data need to be further validated and

tested in preclinical models of SLE and in longitudinal clinical studies.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: https://ega-archive.org/studies/EGAS00001003662.

AUTHOR CONTRIBUTIONS

PG, DN, and SD performed the analyses. PG, SD, and DN drafted the manuscript, with contribution of all authors. GS and Anastasia Filia contributed in figure generation. Antonis Fanouriakis, GB, and EF evaluated clinical data and participated in the analyses and interpretation of the data. DB, GB, and Antonis Fanouriakis conceived, critically revised and oversaw the study and the writing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.860726/full#supplementary-material

Supplementary Figure 1 | Modules (pink to indian-red) of commonly regulated transcripts as identified by CoCena2 analysis and heatmap depicting the group fold changes (GFC) of each sample per module. The identified transcript modules were illustrated in the annotation color bar on the right side of the heatmap. The patients analyzed in our study were shown in the x-axis of the heatmap. GFC were defined for each gene by computing the mean expression of a gene across all samples, followed by calculating the sample specific fold change of the gene expression from the overall mean. Then, the GFC of all genes within each module were added and divided by the total number of genes of each module, returning the GFCs of each sample per module. Briefly, the color intensity represented the relative magnitude of the expression of each gene module per SLE patient. GFC denotes group fold change; GB followed by number denoted the patient identifier according to our anonymous coding system.

Supplementary Figure 2 | Barplots demonstrating the distribution of demographic features as well as the frequency of NPSLE history, Antiphospholipid Syndrome (APS) history, serum anti-DNA antibodies positivity, antiphospholipid antibodies positivity across the patients groups. *:p<0.05; **:p<0.01 in Kruskal-Wallis test, Chi-squared test.

Supplementary Figure 3 | Barplots displaying the treatments the patients were receiving at the sampling timepoint. Cyclophosphamide and MMF were the most commonly used treatments in the G4. *:p<0.05; **:p<0.01 in Kruskal-Wallis test, Chi-squared test.

Supplementary Figure 4 | Clusters of drugs signatures (Cluster 1-4) as identified by k-means clustering according to the ΔNES scores. ΔNES score was defined as the difference between the NES from the downregulated gene set and the NES from the upregulated gene set for each drug signature. Utilizing the calculated ΔNES scores, drug signatures were next grouped using the k-means clustering method into 4 clusters, which were shown on the right side of the heatmap. The heatmap visualized how each of the 4 identified drug clusters were enriched in the specific patient groups. A group specific predominant enrichment of a drug cluster indicated that the drugs included in the drug cluster of interest might be the most potent drug candidates for the specific patient group. Briefly, cluster 4 contained drug signatures that were predicted to most efficiently reverse the transcriptional aberrations of G4. Accordingly, drug cluster 3 might contain the best drug candidates for group G5, whereas drug cluster 1 included drug signatures that might most effectively counteract the G3-specific transcriptional changes.

Supplementary Figure 5 | Elbow method identified optimal number of drug clusters for k-means clustering.

Supplementary Table 1 | Drugs that are currently used in the treatment of SLE or evaluated in SLE clinical trials and their gene expression signatures are included in iLINCS sublibraries.

Supplementary Table 2 | Gene expression signatures of the drugs of table 1. that are listed in the iLINCS prediction databases.

Supplementary Table 3 | Ranking of specific drug related signatures for each SLE patients' molecular endotype, according to ΔNES score.

Supplementary Table 4 | Drug related signatures with ΔNES scores for patient group G4A.

Supplementary Table 5 | Drug related signatures with ΔNES scores for patient group G4B.

Supplementary Table 6 | Ranking of the compounds derived from the iLINCS platform through the CoDReS platform. This analysis was performed for SLE patients' molecular endotypes separately.

Supplementary Table 7 | Ranking of the compounds derived from the iLINCS platform through the CoDReS platform. This analysis was performed for SLE patients' molecular endotypes separately.

Supplementary Table 8 | Ranking of the compounds derived from the iLINCS platform through the CoDReS platform. This analysis was performed for SLE patients' molecular endotypes separately.

Supplementary Table 9 | Ranking of the compounds derived from the iLINCS platform through the CoDReS platform. This analysis was performed for SLE patients' molecular endotypes separately.

Supplementary Table 10 | Ranking of the compounds derived from the iLINCS platform through the CoDReS platform. This analysis was performed for SLE patients' molecular endotypes separately.

Supplementary Table 11 | Ranking of the compounds derived from the CLUE platform, through the CoDReS platform. This analysis was performed for SLE patients' molecular endotypes separately.

Supplementary Table 12 | Ranking of the compounds derived from the CLUE platform, through the CoDReS platform. This analysis was performed for SLE patients' molecular endotypes separately.

Supplementary Table 13 | Ranking of the compounds derived from the CLUE platform, through the CoDReS platform. This analysis was performed for SLE patients' molecular endotypes separately.

Supplementary Table 14 | Ranking of the compounds derived from the CLUE platform, through the CoDReS platform. This analysis was performed for SLE patients' molecular endotypes separately.

Supplementary Table 15 | Ranking of the compounds derived from the CLUE platform, through the CoDReS platform. This analysis was performed for SLE patients' molecular endotypes separately.

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Cross-species transcriptome analysis for early detection and specific therapeutic targeting of human lupus nephritis

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TRANSLATIONAL SCIENCE

Cross-species transcriptome analysis for early detection and specific therapeutic targeting of human lupus nephritis

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ABSTRACT

Objectives Patients with lupus nephritis (LN) are in urgent need for early diagnosis and therapeutic interventions targeting aberrant molecular pathways enriched in affected kidneys.

Methods We used mRNA-sequencing in effector (spleen) and target (kidneys, brain) tissues from lupus and control mice at sequential time points, and in the blood from 367 individuals (261 systemic lupus erythematosus (SLE) patients and 106 healthy individuals). Comparative cross-tissue and cross-species analyses were performed. The human dataset was split into training and validation sets and machine learning was applied to build LN predictive models.

Results In murine SLE, we defined a kidney-specific molecular signature, as well as a molecular signature that underlies transition from preclinical to overt disease and encompasses pathways linked to metabolism, innate immune system and neutrophil degranulation. The murine kidney transcriptome partially mirrors the blood transcriptome of patients with LN with 11 key transcription factors regulating the cross-species active LN molecular signature. Integrated protein-to-protein interaction and drug prediction analyses identified the kinases TRRAP, AKT2, CDK16 and SCYL1 as putative targets of these factors and capable of reversing the LN signature. Using murine kidney-specific genes as disease predictors and machine-learning training of the human RNA-sequencing dataset, we developed and validated a peripheral blood-based algorithm that discriminates LN patients from normal individuals (based on 18 genes) and non-LN SLE patients (based on 20 genes) with excellent sensitivity and specificity (area under the curve range from 0.80 to 0.99).

Conclusions Machine-learning analysis of a large whole blood RNA-sequencing dataset of SLE patients using human orthologs of mouse kidney-specific genes can be used for early, non-invasive diagnosis and therapeutic targeting of LN. The kidney-specific gene predictors may facilitate prevention and early intervention trials.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Prediction of patients with systemic lupus erythematosus (SLE) that will develop nephritis and early diagnosis represents an unmet need because of the limited value of known predictors and the invasiveness of kidney biopsy.

⇒ Even with best treatment up to 40% of patients fail to reach a complete renal response suggesting that early diagnosis and prompt treatment including targeting of renal specific pathways is needed.

WHAT THIS STUDY ADDS

⇒ Distinct, renal-specific molecular pathways are associated with the development of nephritis and its progression from subclinical to full blown disease in murine SLE.

⇒ The mouse kidney transcriptome mirrors the human whole-blood transcriptome in lupus nephritis (LN).

⇒ Upstream and downstream regulators of the cross-species (murine and human) kidney-specific gene signatures have been identified as putative targets in LN and novel cross-species drug signatures for kidney disease in lupus.

⇒ Using the mouse kidney-specific transcriptome and through training by machine-learning techniques of a large whole-blood RNA-sequencing dataset of SLE patients, we developed and validated an algorithm that predicts patients that will develop LN based on a small number (no more than 20) of genes.

INTRODUCTION

In lupus nephritis (LN), current therapy fails to induce remission in more than 50% of patients. Even in cases with clinical remission, repeat kidney biopsies often exhibit residual inflammation and increased fibrosis, with 15%–20% of patients eventually developing end-stage kidney disease.[1–3] Importantly, several clinical trials have failed to meet their primary endpoint[4 5] with only two new treatments approved for LN.[6–9] Accordingly, there is urgent need for therapeutic interventions targeting aberrant molecular pathways enriched within the kidneys, to maximise drug efficacy.

Subclinical (silent) LN represents an early stage in the natural history of the disease[10–12] prior to

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE AND/OR POLICY

⇒ Common cross-species (murine and human) genes could be prioritised as potential therapeutic targets for LN or tested as an alternative, non-invasive 'liquid biopsy' marker of kidney disease in patients with SLE.

⇒ The mouse kidney-specific set of gene predictors may be used towards monitoring human kidney disease in SLE patients and enrolment in LN prevention and early treatment studies.

full-blown disease.[13 14] Notably, genetic and immunological interventions in lupus models have underscored the potential to avert autoantibody deposition and ensuing immune responses within the kidneys,[15–19] suggesting that preemptive therapy might represent a valid therapeutic concept.[15 19] However, the mechanisms underlying the progression to clinical LN are not clearly understood and kidney biopsies at the preclinical stage are not performed.

In this paper, we performed sequential mRNA-sequencing studies in effector (spleen) and target tissues (kidneys, brain) from lupus and healthy mice, as well as in the whole blood of patients with systemic lupus erythematosus (SLE) (including patients with active or responding LN or neuropsychiatric lupus) and healthy individuals. Comparative cross-tissue and cross-species analyses yielded common, cross-species, nephritis-specific genes that could be prioritised as potential therapeutic targets. Using machine-learning algorithms, we constructed a clinical-transcriptome predictive model that can be tested as a non-invasive 'liquid biopsy' marker of kidney disease in patients with SLE, to be used for monitoring of kidney disease in SLE, as well as enrollment in LN prevention and early treatment studies.

METHODS

Patients and healthy individuals

Patients with SLE (n=261) who met the SLICC 2012 or EULAR/ACR 2019 classification criteria and age-matched and sex-matched healthy individuals (n=106) were recruited from the Departments of Rheumatology and Nephrology at the University Hospitals of Heraklio, 'Attikon' University Hospital and the respective Blood Transfusion Units. Active LN was defined by the presence of proteinuria more than 0.5 g/day and active urine sediment. A kidney biopsy was performed in all patients with evidence of active kidney disease. Patients either developed active LN de novo or had had a history of LN and were flaring at the time of sampling. Responding LN was defined by preservation or improvement of kidney function with reduction of proteinuria to less than 50% after 6 months of therapy or less than 0.5–0.7 g/day by 12 months.[20 21] Following informed consent, whole blood was sampled, and RNA was extracted from all participants.

Animals

NZB/W-F1 mice were sacrificed at the prepuberty (1 month old), preautoimmunity (3 months old) and nephritic (6 months old with proteinuria more than 200 mg/dL for three consecutive days) stage of SLE. Age-matched C57BL/6 mice were used as controls. Spleen, kidneys and brain were removed for RNA extraction.

RNA-sequencing

RNA libraries were prepared using the Illumina Truseq kit. Paired-end 37 bp (for mouse) and 67 bp (for human) mRNA-sequencing was performed on the Illumina HiSeq2000 and HiSeq4000, respectively, at the University of Geneva Medical School.[22] FastQC software assessed quality.[23] Raw reads were aligned to the mouse (mm10 version) and human (hg38 version) genome using STAR V.2.6 algorithm.[24] Gene quantification was performed using HTSeq.[25] Differential expression analysis of mouse and human data was conducted using DESeq2[26] and edgeR,[27] respectively. Enrichment and network analyses were performed using gProfiler[28] and GeneMANIA.[29] The Expression2Kinases (X2K)[30] was used to yield transcription factors (TFs), kinases and protein-to-protein interaction (PPI) networks. Prediction of drugs was performed with L1000CDS^2 search engine.[31] Statistical significance was set at 5% false discovery rate (Benjamini-Hochberg).

Machine learning

The human mRNA-sequencing dataset was randomly split into training (70%) and validation (30%) sets. Using the training set and feature selection algorithms, the smallest set of human orthologs that most accurately predicted the outcome of interest was selected. Using these orthologs as predictors, models were fit and compared for their ability to predict human disease. To improve performance, clinical predictors (not included in the definition of active or responding LN) were added to the final model. Accuracy, sensitivity, specificity and area under (AUC) the receiver operating curve (ROC) were determined in the validation set.

Detailed information for all methods can be found in online supplemental material. Scripts used and online supplemental table can be found at https://1drv.ms/u/s!Au_gakpSntTbrGO3-3RQ39ByOId1?e=MLF007.

RESULTS

Molecular signatures associated with murine LN and transition from preclinical to clinical disease

Patients with SLE are in urgent need for therapeutic interventions targeting molecular pathways enriched within individual tissues to treat their disease effectively and safely. To decipher aberrant molecular pathways enriched uniquely within the kidneys in SLE, we profiled gene expression at the spleen (an effector peripheral lymphoid organ), kidneys and brain (major end-organ tissues) from NZB/W-F1 lupus mice and age-matched C57BL/6 healthy counterparts. Tissues were collected at the clinical (nephritic) stage of the disease when nervous system involvement also occurs. Differentially expressed genes (DEGs) in lupus versus healthy mice tissues were analysed. Using genes differentially expressed within kidneys of the NZB/W-F1 lupus mice but not in other tissues studied, we defined a 'kidney-specific signature' comprising 726 DEGs (425 upregulated, 301 downregulated) (online supplemental figure S1A,B, table S1A). Enriched functions within this signature included pathways linked to cell metabolism, innate immune system and neutrophil degranulation (online supplemental figure S1C, table S1B), reiterating the role of neutrophils in lupus kidney injury.[32] By representing the signature DEGs as a gene network, we found several hub genes with high-degree nodes of the network corresponding to human lupus-susceptibility loci[33–35] such as *FCGR2B, PTPRC, ITGAM, NCF1* and *RASGRP1* (online supplemental figure S1D, table S1C).

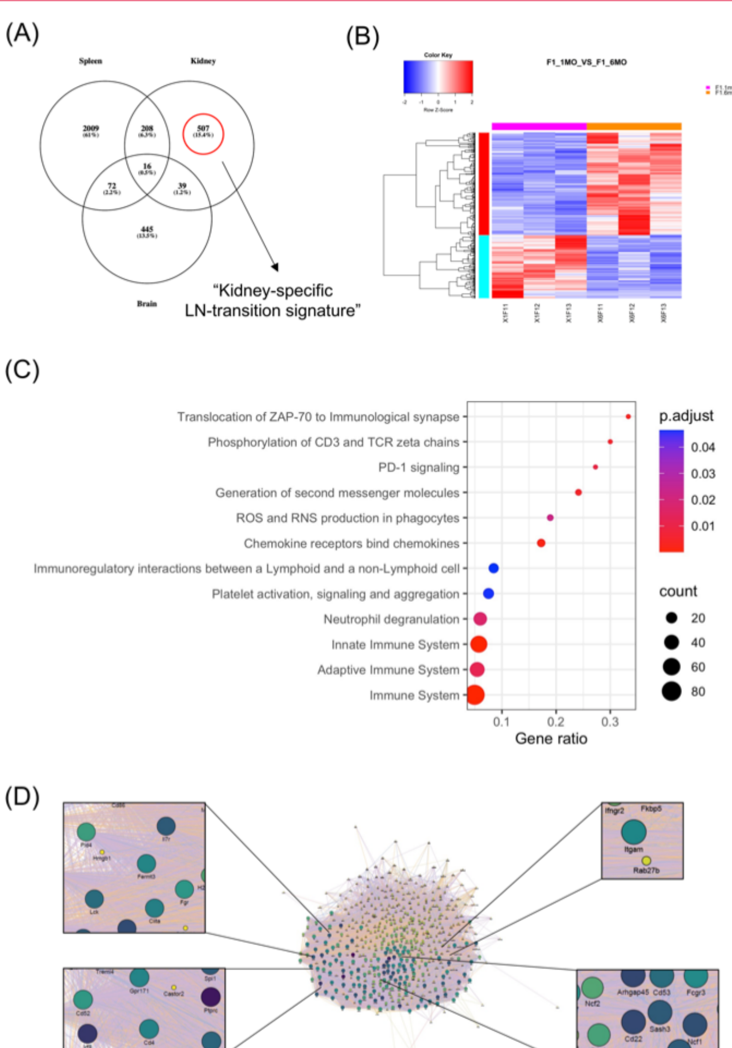


Figure 1 Mouse kidney-specific transcriptome of lupus mice between the clinical (nephritic) and the preclinical (prepuberty) stage of the lupus. (A) Venn diagram demonstrating the comparison between differentially expressed genes (DEGs) within the spleen, the kidneys and the brain from NZB/W-F1 lupus mice at the clinical (nephritic) versus the preclinical (prepuberty) stage of lupus. The kidney-specific gene signature is defined by 507 genes that are differentially expressed only within kidneys but not in other tissues, (B) Heatmap of the 507 kidney-specific DEGs (316 upregulated, 191 downregulated), (C) Dot-plot diagram demonstrating functionally enriched REACTOME pathways of the 507 kidney-specific DEGs, (D) gene network representation of the 507 kidney-specific DEGs. Hub genes that correspond to lupus risk loci are depicted by larger size fonds. ROS, reactive oxygen species; TCR, T cell receptor.

Next, we examined the molecular events underlying transition from the preclinical to clinical stage of lupus kidney disease by comparing DEGs between the tissues from lupus mice probed at the prepuberty versus the nephritic stage. Genes that were differentially expressed uniquely within kidneys of the NZB/W-FI lupus mice but not in other tissues studied defined the 'kidney-specific LN-transition signature' comprising 507 DEGs (316 upregulated, 191 downregulated) (figure 1A,B, online supplemental table S2A) that were enriched in innate and adaptive immune system pathways. The former were linked to neutrophil degranulation and reactive oxygen species production in phagocytes, whereas the latter included T cell receptor signalling, signal transduction by G-protein coupled receptors (in particular, chemokine receptors) and costimulation through programmed cell death protein 1 (PD-1) signalling. In addition, pathways involved in platelet activation, signalling and

aggregation were identified (figure 1C, online supplemental table S2B). Of note, the lupus-susceptibility risk loci *PTPRC*, *NCF1* and *ITGAM* genes, as well as the *IRF8*,[33–35] emerged as hub network genes, suggesting a pathogenic role during evolution from preclinical to clinical LN (figure 1D, online supplemental table S2C).

To analyse the sequential molecular events underlying the evolution towards LN, we identified DEGs in tissues from lupus vs healthy mice demonstrating a strain-specific effect in a time-series analysis. DEGs within kidneys demonstrating the lupus-specific pattern were combined with genes within kidneys that were differentially expressed across all stages of the disease. Combined signatures were compared across tissues and genes that were differentially expressed uniquely within kidneys—but not in other tissues—defined the 'sequential kidney-specific signature', composed of 1668 genes (online supplemental table S3A). Functional interpretation of the result revealed enrichment in the establishment of sister chromatid cohesion pathway (online supplemental table S3B). Kidney-specific DEGs in lupus versus healthy mice at the preautoimmunity stage, kidney-specific DEGs from lupus mice at the preautoimmunity versus the prepuberty stage and the respective functional enrichment analyses are presented in online supplemental tables S3C–F. DEGs within kidneys demonstrating the strain-specific pattern in the time-series analysis are presented in online supplemental figure S2.

The human peripheral blood and the murine kidney transcriptome share common kidney-specific signatures and associated hub genes

Kidney biopsy, an invasive procedure linked to increased risk for adverse events, is currently essential to confirm diagnosis and guide therapeutic decisions in LN; however, it is still an imperfect predictor of response to treatment. Previous studies have reported shared molecular signatures within LN kidneys of mice and humans,[36] as well as between kidney and non-kidney (eg, skin) tissues of patients with LN.[37 38] Recent evidence suggests that neutrophils from ultraviolet skin reach the kidney and cause inflammation in murine models; it is conceivable that these circulating neutrophils prior to their homing to the kidneys may be captured in the blood.[39] To this end, we next asked whether the kidney-specific signatures in murine lupus may exist also in patients with LN using blood as an easily accessible, minimally invasive tissue. Specifically, we investigated whether the mouse kidney could serve as non-invasive (not-requiring biopsy in humans) marker of kidney disease in human SLE. To address this, we performed whole-blood mRNA-sequencing in 141 SLE patients and 48 healthy counterparts. Data were combined with our previously analysed cohort,[22] thus yielding a dataset of 367 individuals (including 261 SLE patients and 106 healthy individuals) (online supplemental table S4A). We found extensive transcriptome perturbations with 10 672 DEGs between active LN patients and healthy individuals (online supplemental figure S3A, table S4B) and 4119 DEGs between active LN and SLE patients without history of kidney disease (non-LN patients) (figure 2A, online supplemental table S4C).

Next, we examined whether the human peripheral blood from patients with LN shares common gene expression aberrations with the mouse kidney-specific gene signatures. Using the human orthologous genes of the mouse genome, we examined if the mouse 'kidney-specific signature' is present in the blood of patients with active LN as compared with healthy individuals. A total 272 genes (193 upregulated and 79 downregulated) were common between the two datasets (online supplemental figure S3B,C, table S5A), referred to as 'shared active LN signature'. Neutrophil degranulation was the most significantly enriched pathway in this signature (online supplemental figure S3D, table S5B), whereas gene network analysis revealed that the lupus-susceptibility risk loci *NCF2, ITGAM, NCF1, RASGRP1* and *FCGR2A*[33–35] were high-degree hub genes, suggesting their central pathogenic role in LN (online supplemental figure S3E, table S5C).

A similar cross-species analysis was performed to determine whether the mouse 'kidney-specific LN-transition signature' intersects with the human blood transcriptome of patients with active LN versus non-LN patients. Ninety-seven common genes (67 upregulated and 30 downregulated) were identified (figure 2B,C, online supplemental table S6A), comprising the 'shared active LN-transition signature'. Functional enrichment analysis revealed pathways linked to hematopoietic cell lineage, B-cell receptor signalling and immunoregulatory interactions between lymphoid and non-lymphoid cell (figure 2D, online supplemental table S6B). *CD53*, *ITGB2* and *LAPTM5* were the highest-degree hub genes, underscoring their role in evolution of LN. The risk locus *ITGAX* was also identified, further supporting its pathogenic role[33] and its gene expression deregulation within kidneys during lupus progression (figure 2E, online supplemental table S6C).

To characterise the 'sequential kidney-specific signature' in the context of human LN, we compared the human orthologous genes of the mouse signature with the DEGs between active LN patients and healthy individuals and revealed 609 common genes that defined the 'shared sequential kidney-specific signature' (online supplemental table S7A). These genes were functionally enriched in pathways linked to selenocysteine synthesis and non-sense mediated decay independent of the exon junction complex (online supplemental table S7B).

In silico analysis of upstream regulators, downstream kinases and drug signatures for the identification of novel therapeutic targets in LN: Kinases TRRAP, AKT2, CDK16 and SCYL1 as putative targets for reversing the LN signature

Genetic association studies have identified TFs to play a major pathogenic role in SLE.[40] Taking advantage of our study design, we performed TF enrichment analysis[30] in the cross-species gene signatures and found a total of 11 TFs (including E2F4, FOXM1, SPI1 and SIN3A) and 6 TFs (including SPI1, IRF8, RUNX1 and VDR), which were predicted to regulate the 'shared active LN signature' (figure 3A, online supplemental table S8A) and the 'shared active LN-transition signature' (figure 3B, online supplemental table S9A), respectively.

To decipher downstream kinases of the shared gene signatures that might serve as druggable targets, the aforementioned lists of enriched TFs were expanded by identifying proteins previously shown to physically interact with them, followed by construction of PPI subnetworks (online supplemental table S8B, table S9B). Based on the overlap between known kinase–substrate phosphorylation interactions and the proteins in the subnetworks, we found kinases that phosphorylate the proteins interacting with the TFs. The kinase TRRAP was predicted to phosphorylate the NCOR2 and HCFC1 (hypergeometric $p=0.0004799$) that interact with the enriched TFs that regulate the 'shared active LN signature' (online supplemental table S8C); and the AKT2, CDK16 and SCYL1 kinases were predicted to phosphorylate ACTN4 and AES or SMARCA4 or AES (hypergeometric $p=0.005443$), respectively, that interact with the enriched TFs that regulate the 'shared active LN-transition signature' (online supplemental table S9C), suggesting they could represent

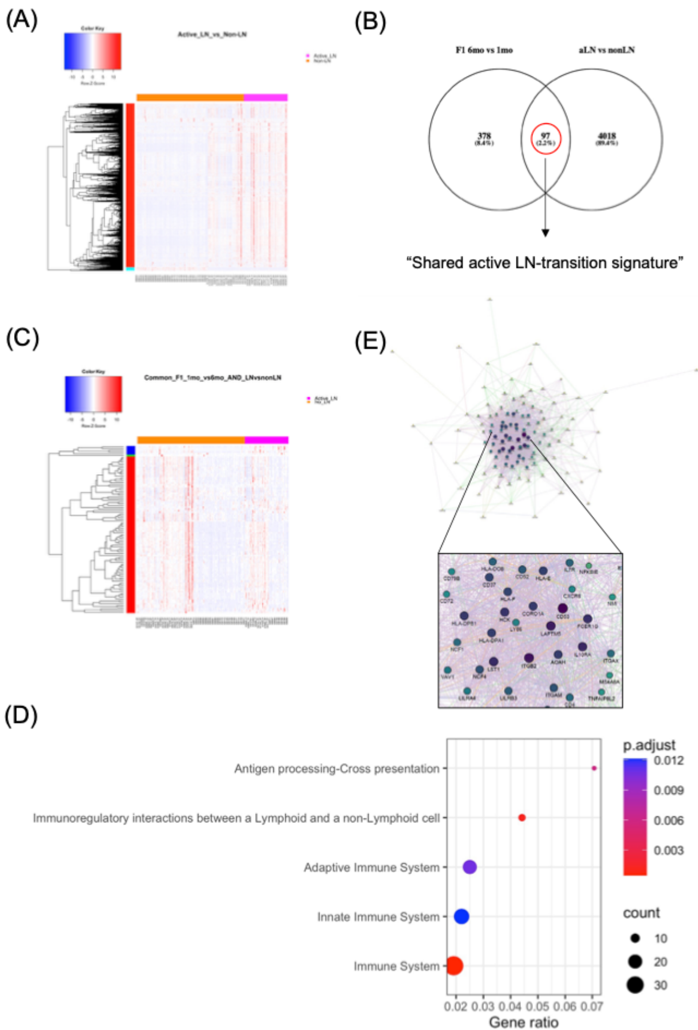
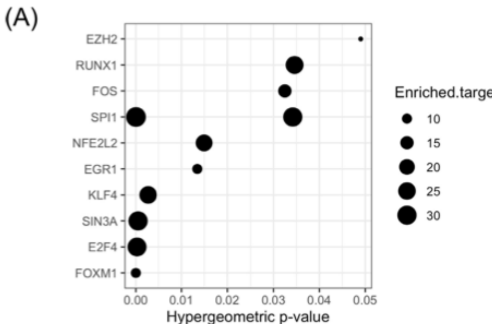


Figure 2 Common genes between the kidney-specific gene expression profile from lupus mice at the symptomatic (nephritic) versus the asymptomatic (prepuberty) stage and the whole-blood gene expression profile from active LN (aLN) patients versus SLE patients without history of kidney involvement (non-LN) define a 'shared active LN-transition signature'. (A) Heatmap of the 4119 differentially expressed genes (DEGs) in the whole-blood from aLN patients versus non-LN patients, (B) Venn diagram demonstrating the comparison between the orthologous genes of the mouse kidney-specific DEGs from NZB/W-F1 lupus mice at the symptomatic (nephritic) versus the asymptomatic (prepuberty) stage and the whole-blood gene expression profile from aLN versus non-LN SLE patients. The 'shared active LN-transition signature' is defined by the union of the Venn diagram, corresponding to 97 common genes, (C) Heatmap of the 'shared active LN-transition signature', composed of 97 genes (67 upregulated, 30 downregulated), (D) Dot-plot diagram demonstrating functionally enriched REACTOME pathways of the 'shared active LN-transition signature', (E) gene network representation of the 'shared active LN-transition signature'. Hub genes that correspond to lupus risk loci are depicted by characters of a larger size. LN, lupus nephritis; SLE, systemic lupus erythematosus.

putative targets in LN. Complete upstream pathways of the gene signatures connecting the enriched TFs to kinases through known PPIs were also inferred (online supplemental tables S8D and S9D).

Finally, through the L1000 Characteristic Direction Signature Search Engine (L1000CDS^2), we detected the top 50 drugs or small molecule compounds (online supplemental tables S8E and S9E) and the top 50 compound combinations that may reverse



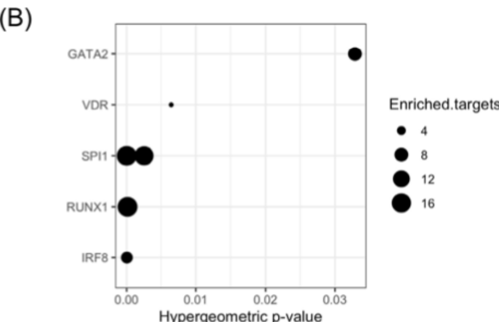


Figure 3 Upstream regulators of the 'shared active LN signature' and the 'shared active LN-transition signature'. (A) Dot-plot diagram demonstrating the transcription factors (TF) that are predicted to reverse the common genes between the kidney-specific gene expression profile from lupus vs healthy mice at the clinical (nephritic) stage and the whole-blood gene expression profile from active LN (aLN) patients vs healthy individuals (HI). The x-axis represents the hypergeometric p value and dots correspond to the number of enriched targets of the TF, (B) Dot-plot diagram demonstrating the TF that are predicted to reverse the common genes between the kidney-specific gene expression profile from lupus mice at the clinical (nephritic) versus the preclinical (prepuberty) stage and the whole-blood gene expression profile from patients with active LN (aLN) versus SLE patients without history of kidney involvement (non-LN). The x-axis represents the hypergeometric p- value and dots correspond to the number of enriched targets of the TF. LN, lupus nephritis; SLE, systemic lupus erythematosus.

the 'shared active LN signature' and the 'shared active LN-transition signature', respectively (online supplemental tables S8F and S9F). Among these, the R(+)−6-BROMO-APB was predicted to reverse the former, and the HEMADO, norketamine hydrochloride, trichostatin A and others were predicted to reverse the latter signature, respectively, in the HA1E kidney cell line, suggesting they could be further tested in the therapy of LN.

Eighteen genes may predict patients with active LN from healthy individuals

Demographic, clinical and serological data are imperfect in predicting the onset of kidney disease in patients with SLE. Importantly, early identification and prompt treatment have been linked to improved outcomes.[13 14] We examined whether the human orthologs of the mouse kidney-specific gene signatures and the human whole-blood gene signatures may predict those patients with SLE who will develop LN. For this, the complete mRNA-sequencing dataset was randomly split into training (70%) and validation (30%) sets, and machine-learning algorithms were applied (figure 4).

To distinguish patients with active LN from healthy individuals, we used the human orthologs of the mouse kidney-specific DEGs from lupus versus healthy mice at the nephritic stage (corresponding to the 'kidney-specific signature', composed of 726 DEGs). To remove noise and keep the smallest set of human orthologs of the mouse genes which best predicts outcome, we performed feature selection using recursive feature elimination with a random forest (machine-learning) model under a 10-fold cross-validation. Based on model accuracy, a set of 50 human orthologs were selected. Next, prediction models were fit to identify which performs best with the selected genes. The glmnet model using 18 genes—including *PLD4*, *PTPRN2*, *CASP8* and *POLE* (figure 5A, online supplemental table S10)—(32 genes had a coefficient=0 and were considered redundant in the model) best distinguished patients with active LN from healthy individuals with a 10-fold cross-validation calculated accuracy of 95.7% (95% CI (0.85% to 0.99%)], 100% sensitivity and 92.9% specificity (0.99 AUC of the ROC curve analysis) in the validation set (figure 5B,C), demonstrating an excellent model efficiency to discriminate true positive (active LN patients) from false positive (healthy individuals) cases. Inclusion of clinical factors (not included in the definition of active or responding LN), such as age, gender and the presence of anti-dsDNA, did not improve further the performance of the model. Using the validation set, principal component analysis (PCA) demonstrated that the 18 selected genes could accurately discriminate patients with active LN from healthy individuals (figure 5D). The relationship between the expression of each gene and the probability of predicting active LN is demonstrated in online supplemental figure S4. These data define a LN prognostic gene signature and demonstrate the feasibility of developing and validating an algorithm to predict patients with active LN from healthy individuals non-invasively, through machine-learning analysis of a large whole blood RNA-sequencing dataset of SLE patients using human orthologs of mouse kidney-specific genes as predictors of kidney involvement.

Machine-learning model distinguishes LN from non-LN SLE patients

Next, we examined whether the above approach could also discriminate active LN patients from SLE patients without kidney disease (non-LN patients) in a non-invasive manner. We sought that the kidney-specific gene expression profile of lupus mice at the clinical (nephritic) versus the preclinical (prepuberty) stage of the disease (corresponding to the 'kidney-specific LN-transition signature', composed of 507 DEGs) could reflect the whole-blood gene expression profile of SLE patients with active LN versus SLE patients without history of LN (non-LN patients). Thus, we used the human orthologs of the mouse 'kidney-specific LN-transition signature' as predictors, and applied feature selection under a 10-fold cross-validation. Based on accuracy, 20 genes best predicted the outcome. Models were fit to identify which performs best with the selected genes. Model performance was further improved by the addition of age, sex and presence of anti-dsDNA, as predictors of outcome. As expected, due to the higher likelihood of patients with proliferative LN to have anti-DNA antibodies, the presence of anti-dsDNA was the most important predictor of kidney disease,

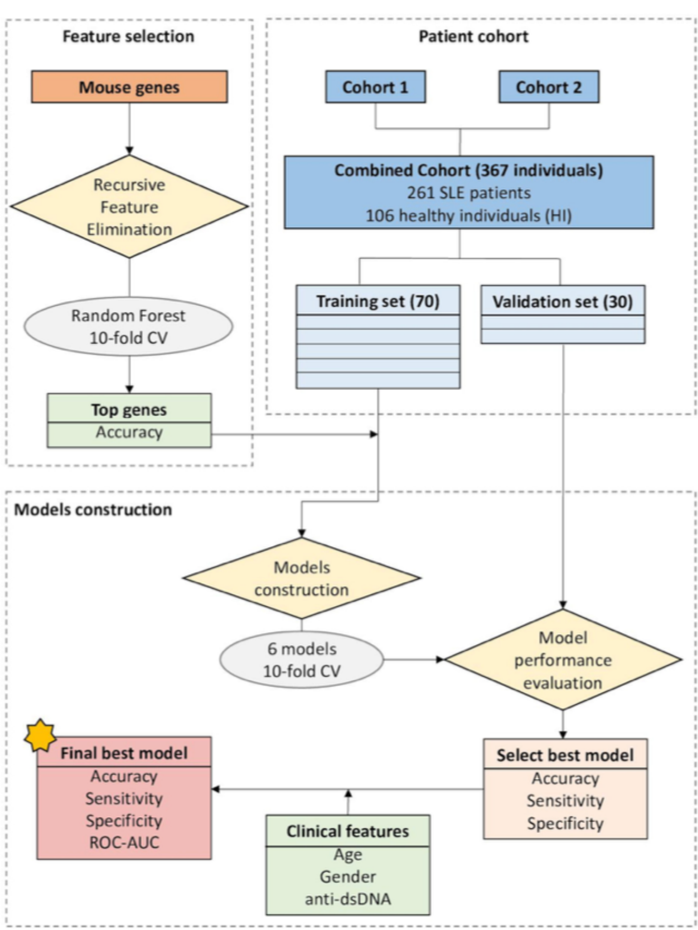


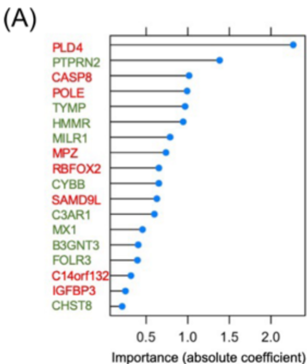
Figure 4 Schematic overview of the machine-learning approach. RNA-sequencing data from the two human cohorts were combined and then split in training to test sets at 70:30 ratio. For each outcome measure, a corresponding gene list derived from mouse data was used. The training set was used to develop a prediction model and the test set was used to validate the results. Using the training set, feature selection was applied to remove noise and keep the smallest set of genes which best predicts each outcome based on accuracy. Then, different prediction models were fit to identify which performs best using the gene signature selected in the previous step. Once the best model was selected based on accuracy, sensitivity and specificity, the addition of age, gender and the presence of anti-dsDNA as predictors were tested if they could improve the model. The final model was validated in the test set. AUC, area under the curve; CV, cross-validation; dsDNA, double-stranded DNA; ROC, receiver operating characteristic curve.

followed by the expression of *PTPRO* gene (the lower its expression, the higher the probability of predicting active LN) and *IL10RA* gene (the higher its expression, the higher the probability of predicting active LN). Male sex and younger age of SLE patients were associated with higher probability of active LN. In the validation dataset, the glm model displayed accuracy 81.7% (95% CI (0.70% to 0.90%)), sensitivity 63.2% and specificity 90.2% (AUC 0.80) in distinguishing patients with active LN from SLE patients without history of LN (figure 6A–C, online supplemental table S11, figure S5), demonstrating that the model correctly identified SLE patients without LN (true negative cases). Using the validation set, PCA demonstrated how gene predictors could accurately discriminate patients with active LN from non-LN SLE patients (figure 6D). Together, these data demonstrate the feasibility to distinguish patients with active LN from SLE patients without kidney involvement. These gene predictors could be of prognostic value in the clinical setting following further validation studies in independent cohorts.

DISCUSSION

Patients with LN are in need for an early diagnosis and therapeutic targeting of aberrant molecular pathways enriched within the affected kidneys. Here, we performed sequential mRNA-sequencing in three tissues of lupus and healthy mice, and in the whole-blood of SLE and healthy individuals. Through cross-tissue analysis, we defined a murine kidney-specific molecular signature and a molecular signature that underlines progression

(A)



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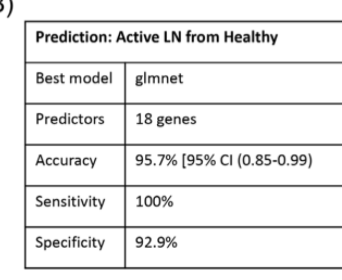
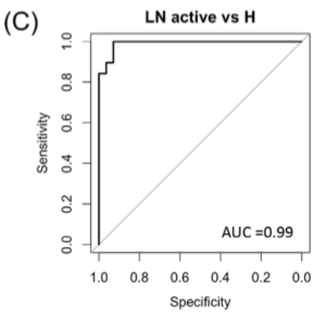


Prediction: Active LN from Healthy	
Best model	glmnet
Predictors	18 genes
Accuracy	95.7% [95% CI (0.85-0.99)
Sensitivity	100%
Specificity	92.9%

(C)



(D)

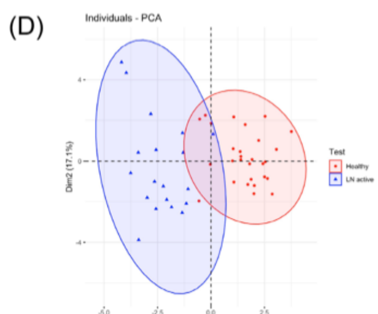
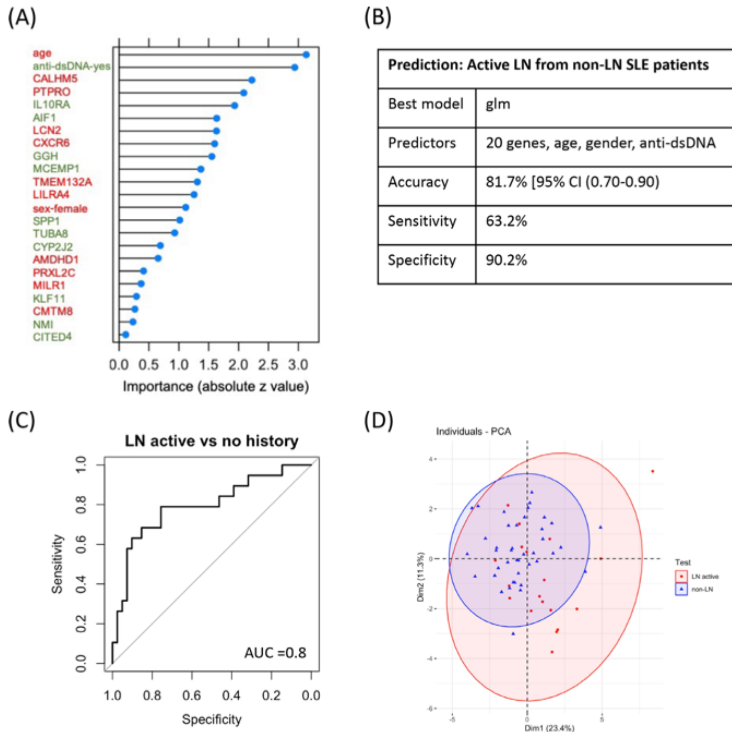


Figure 5 Machine-learning modelling of the human whole-blood RNA-sequencing data, using mouse kidney-specific genes as predictors, distinguishes patients with active lupus nephritis (active LN) from healthy individuals (H) in a non-invasive manner and defines a LN prognostic gene signature. (A) The 18 predictors of the glmnet model distinguishing patients with active LN from healthy individuals based on their importance, as evidenced by their absolute coefficient. Gene predictors in green fonts indicate that the higher their expression the higher the probability of being a patient with active LN compared with being a healthy individual; while gene predictors in red fonts indicate that the lower their expression the higher the probability of being a patient with active LN, (B) Characteristics of the prediction model of patients with active LN from healthy individuals, (C) Receiver operating characteristic curve (ROC) analysis of the glmnet model in the validation set reveals an area under the curve (AUC) of 0.99, (D) principal component analysis (PCA) using the 18 genes.

from the predisease stage to overt clinical disease. We also demonstrated that the murine kidney transcriptome mirrors—in part—the human whole blood transcriptome of LN patients and found upstream and downstream transcriptional regulators that may be prioritised as potential therapeutic targets. Finally, we developed a blood gene-based predictive model for human LN that can be tested as an alternative, non-invasive 'liquid biopsy' marker of kidney disease in patients with SLE. Pending further confirmation, this marker could identify patients in need of monitoring for development of LN, as well as enrolment in LN prevention and early treatment studies.

To improve therapeutic interventions and optimise the use of animal models, gene expression profiling across three samples and species is important in defining how mouse biology can be extrapolated to humans.[41] To this end, the sequential cross-organ (murine spleen, kidney and brain) and cross-species (murine and human) comparative transcriptomics analysis in this paper is novel, defining unique-to-kidney molecular aberrancies in SLE that can be extrapolated to the transition from the preclinical to clinical stage of human LN. Our human transcriptomic analysis involved a large number of well-characterised patients and healthy controls which makes it the larger, single-centre, RNA-seq analysis ever performed in SLE. In addition to providing potential biomarkers for prediction and non-invasive diagnosis and monitoring, our data also reflect biological pathways involved both in the development and clinical transition of LN in a systematic and unbiased manner, without preconceived notions.

In view of the heterogeneity of lupus, we used next-generation sequencing as an unbiased and not requiring a priori hypothesis approach to uncover novel molecular pathways implicated in major end-organ injury in SLE. Initially we performed mRNA-sequencing of a peripheral lymphoid organ (the spleen, that may be used as a surrogate of peripheral blood) and two end-organ tissues (kidneys and brain) from the NZB/W-F1 lupus model at the prepuberty, preautoimmunity and nephritic stage of SLE and identified the molecular profile which is expressed uniquely within kidneys of this model—but not in other tissues studied—and the molecular profile that characterises unique-to-kidney molecular events underlying LN transition from the preclinical to clinical stage of kidney disease. In this process, we identified pathways enriched within each signature and found



Prediction: Active LN from non-LN SLE patients	
Best model	glm
Predictors	20 genes, age, gender, anti-dsDNA
Accuracy	81.7% [95% CI (0.70-0.90)
Sensitivity	63.2%
Specificity	90.2%

Figure 6 Machine-learning modelling of the human whole-blood RNA-sequencing data using mouse kidney-specific LN-transition genes as predictors distinguishes patients with active lupus nephritis (active LN) from SLE patients without history of kidney disease, non-invasively. (A) The 23 predictors of the glm model distinguishing patients with active LN (active LN) from SLE patients without kidney disease (non-LN) based on their importance, as evidenced by absolute z value. Gene predictors in green fonts indicate that the higher their expression the higher the probability of being a patient with active LN compared with being non-LN patient, while gene predictors in red fonts indicate that the lower their expression the higher the probability of being a patient with active LN. The presence of anti-dsDNA (indicated in green fonts) is associated with a higher the probability of being a patient with active LN and the older age and female gender (indicated in red fonts) are associated with a lower probability of being a patient with active LN, (B) Characteristics of the prediction model of active LN patients from non-LN patients, (C) Receiver operating characteristic curve analysis of the glm model in the validation set reveals an area under the curve (AUC) of 0.8, (D) Principal component analysis (PCA) using the 20 gene-predictors. LN, lupus nephritis; SLE, systemic lupus erythematosus;

that hub genes correspond to lupus susceptibility risk loci (such as the *PTPRC, ITGAM, NCF1* and *IRF8* genes), reinforcing their pathogenic role in LN and the progression from preclinical to clinical kidney disease. Validating our results, the *VEGF, TLR2* and *SOCS3* genes were also differentially expressed in the kidneys from NZB/W-F1 mice 9 months old vs 6 months old as well as the kidneys from patients with LN.[36] In agreement with Arazi *et al*,[42] genes such as the *ITGAM* and *FCGR2B* were also differentially expressed in the 'kidney-specific gene signature'. The FPR2, *IL18R1*, *ITGAM* and *NCF4* genes were also differentially expressed in the myeloid lineage from paediatric patients with LN,[43] genes such as the *MDP1*, *PTGR1* and *MX2* were also differentially expressed within the kidneys from LN patients, as assessed by microarrays[44] and genes such as the *TMEM167A, TNFAIP8* and *VCAM1* were also differentially expressed in kidney tubular cells from LN patients.[38]

Blood transcriptome analysis identified similarities as well as differences from the molecular signatures detected within kidneys in patients with LN, underscoring that limitations exist in the use of blood for uncovering kidney disease processes.[42] However, gene expression studies have shown shared inflammatory responses within kidneys between mice and humans with LN,[36] but also shared gene signatures between kidney tubular cells and keratinocytes of LN patients.[37 38] Our data suggest that the mouse kidney transcriptome and the human whole-blood transcriptome share a common gene expression profile that corresponds to common biological processes and pathways. Lupus medications were held for 12 hours prior to sampling thus, a potential downstream effect cannot be excluded. However, validating our results, in the 'shared active LN signature', genes such as the *CEACAM1, TYMP, NCOA7* and *AIM2* were also differentially expressed in interferon stimulating genes identified through single-cell RNA-sequencing within the kidneys from LN patients[42] and *SERPINA1, IL1RN* and *ABCB1* genes were also differentially expressed in kidney tubular cells from LN patients.[38] We also identified hub genes of the common cross-species kidney-specific gene network corresponding to lupus-susceptibility risk loci, uncovering their cross-species pathogenic

role in LN, and identified that the pathway interactions between lymphoid and non-lymphoid cell characterises the transition from preclinical to clinical LN across species. Although we do not validate the LN blood transcriptome with the kidney transcriptome in humans, part of the mouse kidney transcriptome mirrors the human whole-blood transcriptome in patients with LN, suggesting that common genes can be prioritised as potential therapeutic targets for LN, or tested as an alternative, non-invasive 'liquid biopsy' marker of kidney disease in patients with SLE.

To decipher cross-species specific targets in LN, we used systems biology approaches and combined our experimental data with simulation-based analyses. We report upstream and downstream regulators of the cross-species kidney-specific gene signatures as specific targets in LN and describe novel cross-species drug signatures for kidney disease in lupus, suggesting non-immune-based approaches to be tested in LN therapeutics, as 'add on' therapy to conventional immune therapy. We must underscore that due to limitations in the analysis, identified TFs are not restricted to immune cells therefore therapies targeting them could have off-target effects with potential toxicity.

Although current therapeutic decisions in LN are guided by its histological classification,[20 21 45] kidney histology is an imperfect predictor of kidney outcome,[1] highlighting the need for improved biomarkers.[44] The urokinase-type plasminogen activator receptor and the decrease in urinary epidermal growth factor to creatine ratio have been identified as independent predictors of progression to chronic kidney disease in patients with glomerular diseases[46 47]; however, a biomarker for preclinical LN has not been identified. Since preclinical LN is an early stage in the natural history of the disease and improvements in the prognosis of LN have been attributed to early diagnosis and prompt therapy,[10–14] we used machine-learning approaches to identify non-invasive predictors of kidney involvement in SLE patients. Specifically, we used the 'kidney-specific gene signature' as a tool to build a machine-learning algorithm to distinguish patients with active LN from healthy individuals and demonstrated that this approach can be used successfully as a non-invasive prediction method. Then, using the murine lupus kidney-specific transcriptome, we built and validated a machine-learning algorithm that predicts patients with active LN from SLE patients without LN, to be used in the monitoring for kidney disease in such patients and enrolment in LN prevention and early treatment studies. Although validation in an independent dataset was not used, cross-validation was performed during modelling, thus reinforcing our results. These gene predictors could be of prognostic value in the clinical setting, following further validation studies in independent cohorts. Although machine-learning distinguishes patients with LN from non-LN patients accurately, yet at this point this method is not better than clinical diagnosis of LN. Moreover, sequential clinical and transcriptomic data are necessary for the prediction of patients that will flare. The prediction of patients that truly have responding LN would have also been useful; however, a kidney-specific signature corresponding to responding kidney disease (not preclinical) is not available in murine, making this algorithm not applicable for this purpose. Further validation in independent human datasets or longitudinal studies are needed to further explore these findings in human LN.

In conclusion, common cross-species, nephritis-specific genes could be used as potential therapeutic targets for LN or tested as a surrogate, non-invasive 'liquid biopsy' marker of kidney disease in patients with SLE. These kidney-specific genes can be used to design prevention and early intervention trials, following their validation in longitudinal studies.

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Competing interests ED is an employee of GSK. His contribution was performed before he joined GSK.

Patient and public involvement Patients and/or the public were not involved in the design, or conduct, or reporting, or dissemination plans of this research.

Patient consent for publication Not applicable.

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Restoration of Aberrant Gene Expression of Monocytes in Systemic Lupus Erythematosus via a Combined Transcriptome-Reversal and Network-Based Drug Repurposing Strategy

Restoration of Aberrant Gene Expression of Monocytes in Systemic Lupus Erythematosus via a Combined Transcriptome-Reversal and Network-Based Drug Repurposing Strategy

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Keywords: systemic lupus erythematosus, monocytes, drug repurposing, micro RNAs, transcription factors.

Abstract

Objectives: Monocytes -key regulators of the innate immune response- are actively involved in the pathogenesis of systemic lupus erythematosus (SLE). We sought to identify novel compounds that might serve as monocyte-directed targeted therapies in SLE.
Methods: We performed mRNA sequencing in monocytes from 15 patients with active SLE and 10 healthy individuals. Disease activity was assessed with the Systemic Lupus Erythematosus Disease Activity Index 2000 (SLEDAI-2K). Leveraging the drug repurposing platforms iLINCS, CLUE and L1000CDS^2, we identified perturbagens capable of reversing the SLE monocyte signature. We identified transcription factors and microRNAs that regulate the transcriptome of SLE monocytes, using the TRRUST and miRWalk databases, respectively. A gene regulatory network, integrating implicated transcription factors and microRNAs was constructed, and drugs targeting central components of the network were retrieved from the DGIDb database.
Results: Inhibitors of the NF-κB pathway, compounds targeting the heat shock protein 90, as well as a small molecule disrupting the Pim-1/NFATc1/NLRP3 signaling axis were predicted to efficiently counteract the aberrant monocyte gene signature in SLE. Based on our network-based drug repurposing approach, an IL-12/23 inhibitor and an EGFR inhibitor may represent potential drug candidates in SLE.
Conclusions: Application of two independent - a transcriptome-reversal and a network-based -drug repurposing strategies uncovered novel agents that might remedy transcriptional disturbances of monocytes in SLE.

Introduction

Monocytes and macrophages constitute a major cellular compartment derived from hematopoietic myeloid precursors. Monocyte-macrophage lineage cells exhibit versatile immunoregulatory, inflammatory and tissue repairing capabilities and play an instrumental role in the development of systemic lupus erythematosus (SLE) [1]. Data from murine and human SLE studies demonstrated that the polyclonal B cell hyperreactivity, an immunological hallmark of SLE, might be at least partially attributable to aberrations in monocyte-mediated CD40/CD40L co-stimulation [1-5]. Abnormal activation of autoreactive T and B cells in SLE could also be caused by dysregulated cytokine production by monocytes. Monocytes in SLE display excess production of the B-lymphocyte stimulator (BLyS) which promotes the survival and proliferation of B cells [6]. Moreover, these cells are a major source of IL10 and IL6 in the peripheral blood of SLE patients, which in turn augments antibody production and induces plasma cell differentiation, respectively [6]. Besides their contribution to the aberrant activation of adaptive immune system, defects in non-inflammatory phagocytosis by macrophages are implicated in the impaired clearance of cellular debris, that serves as a crucial trigger for the production of autoantibodies in SLE [1, 7, 8, 9, 10]. Notably, monocytes in SLE not only significantly contribute to the generation of the interferon (IFN) signature *per se*, but also give rise to plasmacytoid dendritic cells which are considered as the primary type I IFN producing cells in SLE [11,12].

Several powerful computational tools have facilitated *de novo* drug development and drug repurposing processes in a cost-effective and time-saving manner. The library of integrated network-based cellular signatures (LINCS) L1000 dataset integrated over a million gene expression profiles of human cell lines before and after exposure to more than 20,000 perturbagens. Taking a step forward, the LINCS L1000 Characteristic Direction Signatures Search engine (L1000CDS^2) enabled the prioritization of thousands of small-molecule signatures, according to their ability to counteract disease specific transcriptional profiles [13]. We have previously employed an iLINCS-based drug repurposing pipeline [14, 15], suggesting the potential therapeutic relevance of compounds targeting the PI3K/mTOR pathway in SLE.

Herein, we employed two independent drug repurposing approaches to identify novel compounds that might restore the molecular aberrancies of monocytes in SLE. Using the iLINCS, CLUE and L1000CDS^2 platforms, we propose putative novel drugs potentially capable of reversing the monocyte-specific SLE gene signature. We also report FDA-approved drugs and patented compounds that might disturb the gene regulatory network of SLE monocytes, suggesting they should be tested as monocyte-targeted therapies in SLE.

Materials and Methods

Patients

Monocytes were isolated (CD14$^+$ cells through FACS technology, BD FACS ARIA IIu) from peripheral blood samples of 15 SLE patients fulfilling the 2019 EULAR/ACR classification criteria for SLE [16]. Patients were recruited from the Rheumatology Outpatient Department of the Attikon University Hospital and the University Hospital of Heraklion [16]. Ten age- and sex-matched healthy individuals were used as controls. Disease activity was evaluated using the modified Systemic Lupus Erythematosus Disease Activity Index 2000 (SLEDAI-2K); SLEDAI-2K ≥4 defined active disease [17, 18]. All participants provided informed consent and the study approval was obtained from the local institutional review boards.

RNA sequencing and differential expression analysis

RNA libraries were prepared using the Illumina TruSeq kit. Paired-end mRNA sequencing was performed on the Illumina HiSeq2000 platform. The reads were aligned to the human reference genome (GRCh38.p12) by STAR RNA-Seq aligner [19]. Differential expression analysis was conducted using the edgeR Bioconductor R package [20].

Drug repurposing analysis

Using the iLINCS [21], CLUE [22] and L1000CDS^2 [13] platforms, we identified compounds that reverse the SLE monocyte signature. The following libraries were used for search in the iLINCS platform: a) iLincs chemical pertubagen library (LINCSCP); b) Connectivity map signatures (CMAP); c) Drug matrix signatures (DM); d) Cancer therapeutics response signatures (CTRS); and e) Pharmacogenomics transcriptional signatures (PG). Through extensive Pubmed literature review, the top-ranked compounds derived from each platform, were re-evaluated based on their functional relation to SLE-associated gene or protein targets.

Network analysis

The transcription factors and the microRNAs (miRNAs) that regulate the expression of the statistically significant, differentially expressed protein-coding genes were identified using the databases TRRUST and miRWalk, respectively. The drug-protein interactions were retrieved from the DGIdb database. Networks were constructed using the igraph package and their visualizations using the ggraph and qgraph packages in R [23, 24].

Results

The SLE monocyte gene signature can be utilized to predict potential drug repurposing

To propose existing FDA-approved or investigational compounds that might serve as novel monocyte-targeted therapies in SLE, we sought to identify compounds with potency to reverse the monocyte gene expression profile. Differentially expressed genes (DEGs) (absolute Fold Change ≥ 1.5, P-value ≤ 0.01) of monocytes between SLE patients and healthy individuals defined the monocyte-specific signature (Supplementary Table 1). Using iLINCS, CLUE and L1000CDS^2 platforms, the top 50 compounds that were predicted to counteract the SLE monocyte-specific gene signature most efficiently - according to their inhibitory scores - were identified (Supplementary Table 2-4).

Our analysis indicated several p38 MAP kinase inhibitors, such as the "L-skepinone" [25], as a potential novel strategy of tuning monocytes in SLE. Additionally, the mTOR inhibitor "sirolimus" [26], as well as the calcineurin inhibitor "tacrolimus" [27], were recognized as potent modulators of the lupus monocyte gene signature. In line with studies underlying the crucial role of NF-κB in the survival and activation of monocytes [28], NF-κB pathway inhibitors, such as the compound "parthenolide" [29,30], were predicted to reverse the SLE monocyte gene signature, whereas agents targeting the SLE-related Pim-1/NFATc1/NLRP3 signaling axis [31] might also represent promising therapeutic approaches. The sphingosine-1 phosphate receptor modulator "fingolimod", which has shown possible efficacy in neuropsychiatric lupus manifestations in the MRL/*lpr* lupus mouse model [32], might therapeutically interfere with the monocyte-mediated orchestration of immune responses in SLE.

Finally, common compounds reversing the monocyte gene signature were identified by the three different platforms (Figure 1): the heat shock protein 90 inhibitors "geldanamycin" and "NVP-AUY922", the Insulin-like growth factor 1 receptor (IGF-1R) inhibitor "BMS-536924", the BCR-ABL and Src family tyrosine kinase receptor inhibitor "dasatinib" and the Cyclin-Dependent Kinase 9 inhibitor "alvocidib", suggesting they could be further tested as agents reversing the pathological molecular phenotype of monocytes in SLE.

Gene interaction network analysis as a guide for drug repurposing

Next, we sought to propose compounds that modulate the expression of multiple targets in the gene regulatory network of SLE monocytes. To this end, the transcription factors that regulate the transcriptional landscape of monocytes in SLE were retrieved from the TRRUST

database (Supplementary Table 5). To reveal post-transcriptional regulators, the miRNAs that could regulate the gene expression profile of SLE monocytes were yielded using the miRWalk database (Supplementary Table 6). Thus, a comprehensive miRNA-gene interaction network - inferred using the monocyte gene signature, transcription factors and miRNAs - was constructed (Figure 2). Topological analysis of the constructed network uncovered a high degree of interconnectivity of genes encoding the proinflammatory mediators IL6 and IL1β. In line with studies underscoring the pivotal contribution of monocytes as IFN-producing cells in SLE, genes linked to type I IFN pathway, such as *IRF7*, *IFIT3,* as well as the transcription factor *STAT1* emerged as hub nodes [33]. Top-ranked hub miRNAs included the miR-124-3p, which has been found significantly upregulated in peripheral blood mononuclear cells and serum from SLE patients [34], as well as several miRNAs, with still largely unknown function in the context of SLE, such as miR-24-3p, miR-302c-3p and miR-302d-3p.

To identify agents with potentially unrecognized efficacy in SLE, we next determined drugs targeting hub genes of the miRNA-gene interaction network. Using the DGIdb database, a detailed drug-gene interaction network was constructed (Figure 3A, Supplementary Table 7), revealing the anti-IL-12/IL-23 antibody "ustekinumab" and the epidermal growth factor receptor (EGFR) inhibitor "cetuximab". Interestingly, the recombinant human TNF receptor Fc fusion protein "etanercept" as well as the chimeric monoclonal anti-TNFa antibody "infliximab" were identified as highly interconnected nodes.

Considering the extensive alterations of transcriptional regulation in SLE monocytes, we additionally constructed the drug-transcription factor interaction network (Figure 3B). The proteasome inhibitor "bortezomib" was yielded as potential drug candidate, whereas several natural compounds and plant extracts, such as "resveratrol", "quercetin" and "curcumin" might efficiently modulate the activity of the dysregulated transcription factors in SLE monocytes [35-39].

Discussion

Herein, we applied a transcriptome-reversal combined with a network-based drug repurposing approach to identify novel compounds which might represent putative therapeutic options in SLE, through targeting transcriptional disturbances of monocytes. Using high-throughput drug repurposing tools, we identified agents predictive of reversing the molecular aberrations of SLE monocytes. By employing a gene network-based analysis, we propose agents to target essential regulators of the monocyte transcriptional landscape.

Several *in silico* drug repurposing studies have deployed whole blood gene expression profiling to suggest tailored SLE treatment choices [14, 40]. In view of the central role of monocytes in several aspects of SLE pathogenesis [1], it is tempting to speculate that the targeted therapeutic manipulation of monocytes in SLE might improve clinical outcomes and minimize side effects. To this end, we performed a monocyte-specific drug repurposing analysis in the context of SLE. The inhibitor of the serine/threonine kinase Pim-1 "SGI-1776" was identified as a promising monocyte targeted therapy, corroborating experimental data which suggest that inhibition of the Pim-1/NFATc1/NLRP3 pathway ameliorates nephritis in lupus mouse models [31]. Despite the recently published phase 3 trial [42, 43], our findings indicate that the IL12/IL23 inhibitor ustekinumab may efficiently disrupt the molecular interaction network of monocytes and therefore some patients might indeed benefit from this drug.

Previous *in vitro* and *in vivo* data support the notion that HSP90 might represent a potential drug target in SLE [44-46]. Interestingly, HSP90 facilitates the TLR7/9-mediated nucleic acid recognition in SLE, therefore promoting IFN-α production from plasmacytoid dendritic cells [44]. To this end, the potential therapeutic application of the HSP90 inhibitor, geldanamycin, revealed by our analysis could merit further clinical investigation.

Complete understanding of miRNA regulation in SLE still remains elusive. Herein, we detected novel miRNAs, which might possess regulatory properties in the gene network of SLE monocytes. Given that each miRNA could concurrently influence multiple effectors of pathways, targeting the dysregulated miRNAs may also show promise for the future treatment of SLE. Accordingly, therapeutic modulation of the highly interconnected miR-124-3p, which has been designated as predictor of remission in SLE [34], might shed new insights into SLE treatment.

Our study has certain limitations, related to the function and topology of the cell subset and the methods used. Tissue macrophage compartment in steady state is mainly derived from embryonic precursors and actively contributes to maintenance of tissue homeostasis and resolution of inflammation [41]. Therefore, targeted pharmacological manipulation of tissue resident macrophage populations that might be driving pathology in SLE needs to be evaluated. In addition, our analysis is a computational approach and further experimental and clinical investigation is required to validate our findings.

In summary, using two independent computational system biology approaches, we identified novel compounds that are predicted to restore the function of monocytes in SLE. The

therapeutic implications of our findings need to be further defined in animal models of SLE models and then tested in clinical trials.

Figures

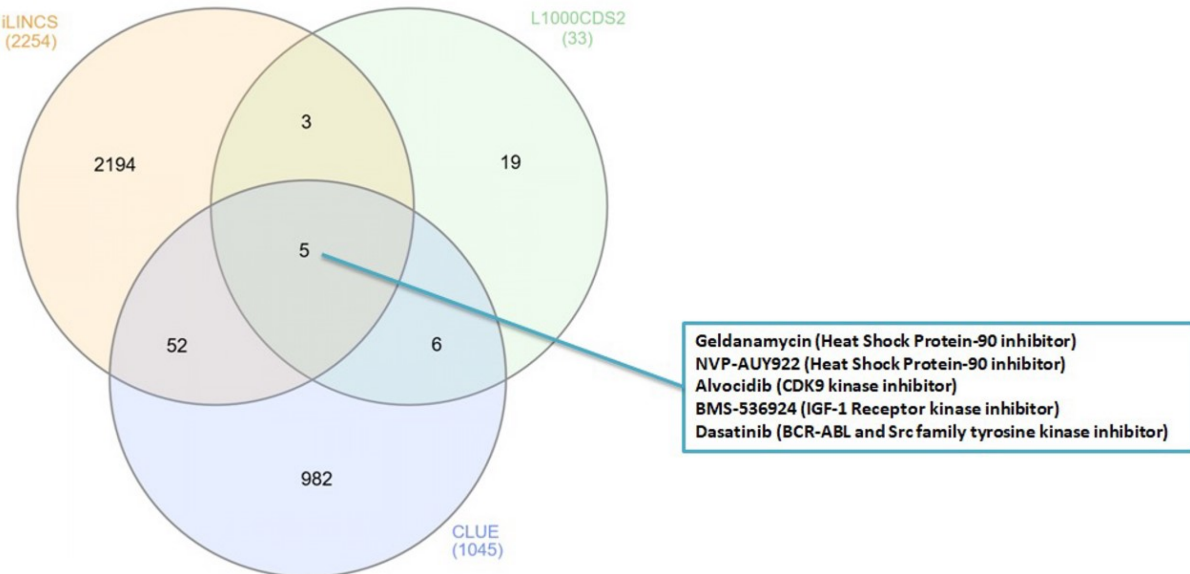

Figure 1. Venn diagram demonstrating the common compounds identified by the three different drug repurposing platforms, iLINCS, CLUE and L1000CDS^2.

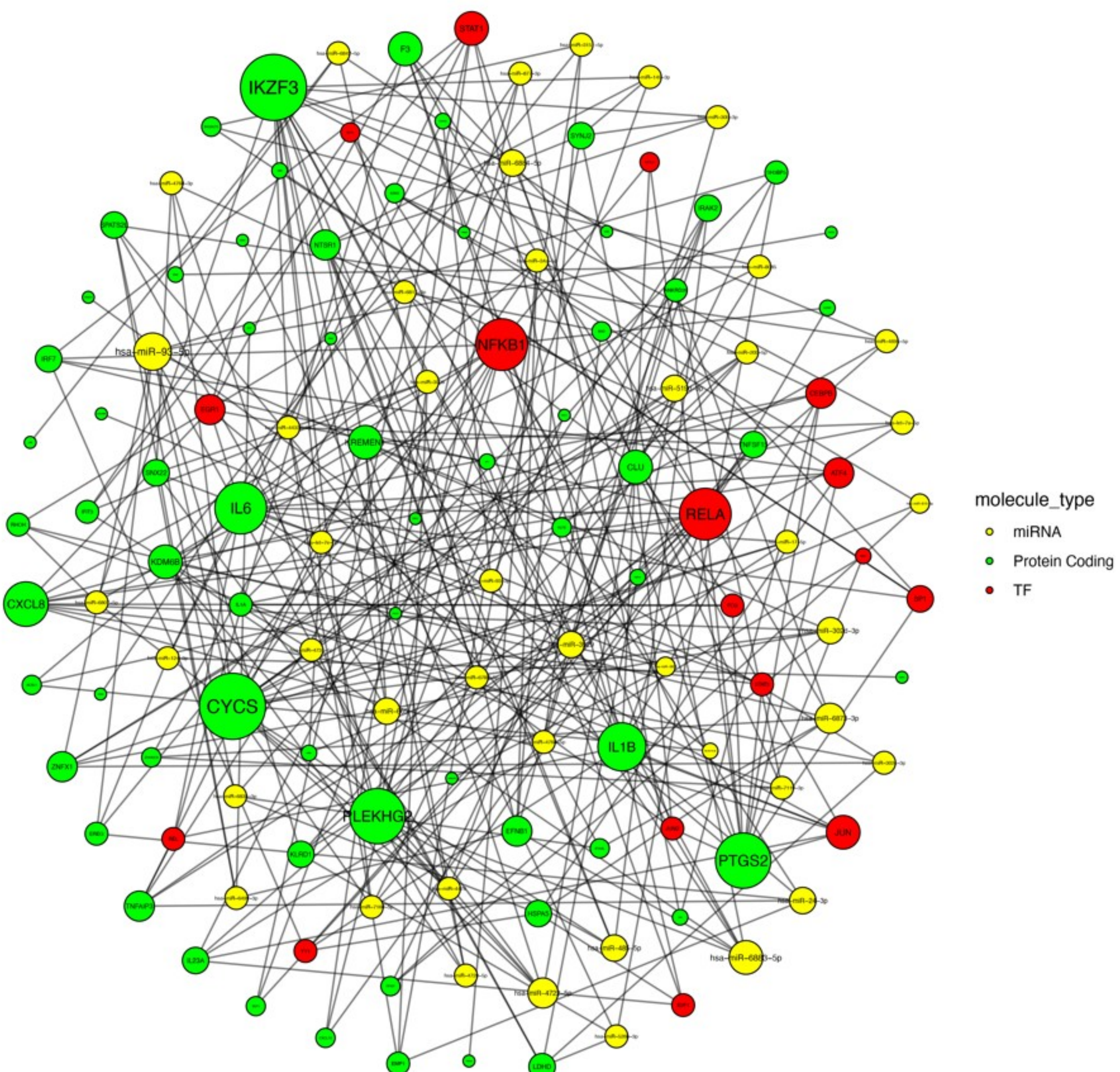

Figure 2. Interaction network integrating the protein coding differentially expressed genes (DEGs) identified by the differential expression analysis of the monocytes from SLE patients versus healthy individuals, the transcription factors identified to regulate their expression and the miRNAs that are associated with them. Only nodes with degree >3 were depicted. Genes encoding the interleukins IL6, IL1b as well as genes implicated in the JAK/STAT pathway were among the most highly interconnected nodes.

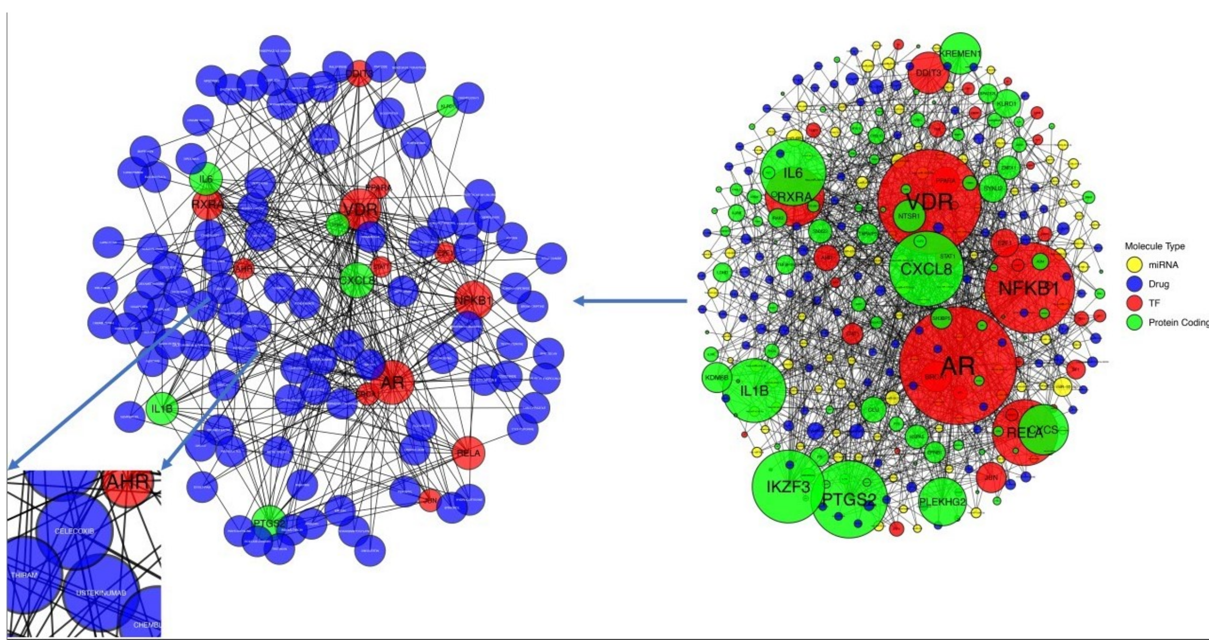

Figure 3A. Interaction network combining the protein coding differentially expressed genes (DEGs), the transcription factors and the miRNAs as defined in Figure 2 and the drugs that are predicted to interact with the DEGs, according to the DGIdb database. Nodes with degree > 2 were included in the network on the right side of the graph. From the nodes included in the network on the right side of the graph, we selected the DEGs, transcription factors and miRNAs with degree > 10, as depicted in the network on the left side of the graph. Among others, the monoclonal antibodies targeting the IL12/IL23 as well as the TNF pathways were identified.

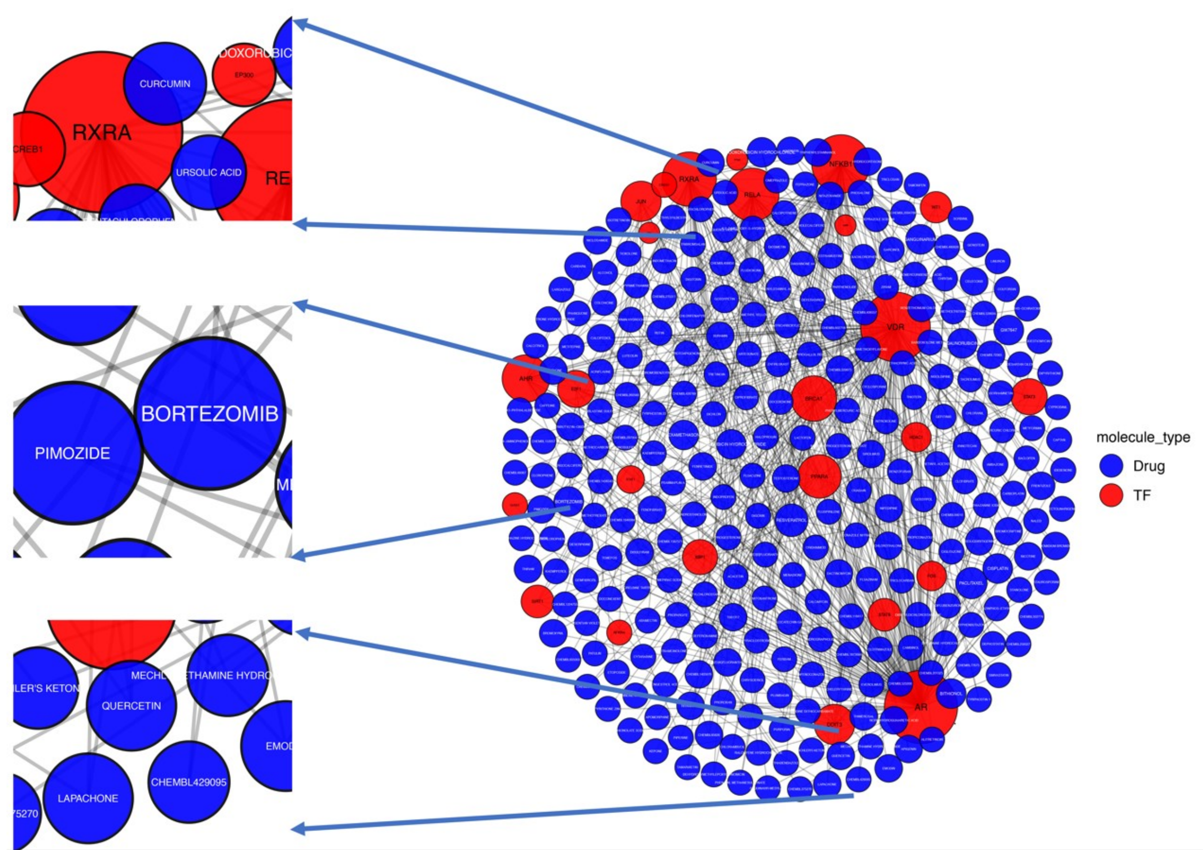

Figure 3B. Interaction network showing the transcription factors that regulate the expression of the monocyte gene signature in SLE and the compounds that interfere with their function. Only nodes with degree > 2 were demonstrated. The proteasome inhibitor bortezomib as well as several natural products emerged as potential drug candidates.

Author Contributions

D.N.(Dimitrios Nikolakis): Conceptualization, experiments, data analysis, writing-original draft preparation, review and editing

P.G.: Conceptualization, experiments, data analysis, writing-original draft preparation, review and editing

G. S.: Visualization, data analysis, editing

A.F.: Review and editing

G.B.: Review and editing

E.F.: Editing

D.N.(Dionysis Nikolopoulos): Patient Recruitment

A.B. : RNA sequencing experiments and editing

D.B.: Supervision, review and editing

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Supplementary Material

Supplementary Material (Supplementary Tables)

Supplementary Table 1. List of the statistically significantly differentially expressed genes (DEGs) resulting from the comparison of the monocytes from SLE patients versus healthy controls.

Supplementary Table 2. List of Compounds that are predicted to reverse the monocyte signature in SLE, derived from the iLINCS platform-based drug repurposing analysis.

Supplementary Table 3. List of Compounds that are predicted to reverse the monocyte signature in SLE, derived from the CLUE platform-based drug repurposing analysis.

Supplementary Table 4. List of Compounds that are predicted to reverse the monocyte signature in SLE, derived from the L1000CDS^2 platform-based drug repurposing analysis.

Supplementary Table 5. List of the transcription factors that are predicted to regulate the expression of the monocyte gene signature in SLE, retrieved from the TRRUST database.

Supplementary Table 6. List of the miRNAs that are predicted to regulate the expression of the monocyte gene signature in SLE, retrieved from the miRWalk database.

Supplementary Table 7. Topological analysis of the interaction network of Figure 3A.

Discussion

SLE is heterogenous, multisystem autoimmune disease with paroxysmal and largely nonlinear disease course. Although the prognosis and the survival of SLE patients have been dramatically improved over the last decades, management of SLE still remains far from optimal. To this end, integration of personalized medicine into clinical practice could advance research endeavors and yield remarkable progress in treatment of SLE patients. In this study, we employed a robust molecular taxonomy strategy in order to re-stratify patients with SLE based on their whole blood transcriptional fingerprints. Leveraging the molecular endotypes determined by the co-expression network analysis, we established an *in-silico* drug prediction pipeline to select compounds tailored to each group's molecular portraits. To identify novel, potentially beneficial therapeutic agents, that could restore the whole blood molecular disturbances of SLE patients, we also applied a transcriptome-reversal drug repurposing strategy. Finally, considering the instrumental role of the monocytes in the development of SLE, we employed a combined transcriptome-reversal and network-based drug repurposing strategy in order to propose patented compounds, that might efficiently target multiple genes of the transcriptional landscape of the SLE monocytes.

The heterogeneity of SLE confounds the diagnosis and the treatment of the disease. Specifically, the clinical and molecular diversity of the disease often accounts for the great variability in response to treatment, hindering effective drug development in SLE. Many transcriptional studies in the last few years have focused on the data-driven stratification of the SLE patients, to guide precision care and inform clinical trial design. For example, type I IFN [**22**], granulopoiesis-related [**21**], as well as CD8+ T cell exhaustion gene expression signatures [**71**] could define SLE endotypes according to disease susceptibility, activity, and severity, while machine learning-based approaches, leveraging gene expression data could predict SLE disease activity with 70% accuracy [**72**]. In the same context, a longitudinal analysis of a well-characterized paediatric cohort of patients with SLE identified the functionally annotated molecular endotypes: plasmablast, type I IFN response, neutrophil/myeloid cell, and lymphocyte [**23**]. Additionally, single-cell transcriptomic analyses of renal, skin biopsies, blood and urine from LN patients resolved differential cellular responses and provided novel insights into pathophysiological mechanism underlying SLE at tissue level [**73-75**].

Herein, we re-stratified the SLE patients, using one of the largest, single-center RNA sequencing cohorts, including rich phenotyping data. Notably, our taxonomy strategy recapitulated the whole spectrum of pathophysiological features underlying SLE. For example, gene expression signatures indicative of neutrophil activation and degranulation defined the patient group 4, which included almost uniformly patients with active LN, corroborating experimental data suggesting that progression to active lupus nephritis is accompanied by an incremental enrichment of neutrophilic gene expression signatures [**23**]. Importantly, contrary to previous studies, the scope of our study was not limited in the molecular taxonomy in SLE, but rather included a signature-based drug prediction analysis. Specifically, exploiting the drug signature databases of iLINCS, we proposed endotype-tailored therapeutic options from a pool of currently available drugs.

With few notable exceptions, the novel insights into pathogenesis of SLE have failed to translate into new therapies. Given the substantial costs, the existing limitations in SLE trial design, the high attrition rates and the slow pace of drug discovery and development, re-inventing approved or abandoned compounds by screening them for new indications has emerged as an attractive proposition. Herein, using two robust, high-throughput platforms (iLINCS and CLUE), we identified novel agents that could target the PI3K/Akt/mTORC1 and the JAK/STAT pathways and might represent potential endotype-specific drug candidates. Since monocytes have been implicated as key players in the pathogenesis of SLE, we additionally applied two independent computational system biology approaches to propose agents that might reverse the transcriptional disturbances of SLE monocytes. In accordance with previous studies indicating that pharmacological dampening of the Pim-1/NFATc1/NLRP3 pathway ameliorates nephritis in lupus mouse models, our analysis proposed the inhibitor of the serine/threonine kinase Pim-1 “SGI-1776” as a potential monocyte targeted therapy in SLE.

Highly complex molecular stratification strategies do not undermine the importance of a unifying, core gene expression signature, that could assist diagnosis in SLE. To this end, Haynes et al. performed an integrated, multi-cohort meta-analysis of 7,471 samples from 40 independent, publicly available whole transcriptome SLE datasets [**76**]. A 93-gene signature, consistent across diverse tissues and cell types, efficiently distinguished SLE from other autoimmune or inflammatory diseases and correlated significantly with disease activity [**76**]. Herein, applying a comparative cross-tissue, cross-species, time-series analyses, we

determined a LN specific gene signature, that might serve as a surrogate, non-invasive "liquid biopsy" marker of kidney disease in patients with SLE.

Our study has several limitations. Firstly, the observational nature of our analysis, does not allow the detection of predictors of flare. The majority of the patients included in our study were receiving immunosuppressive agents, suggesting that treatment-induced transcriptional alterations might have an impact on our results. Additionally, our study is a computational approach, and further *in vitro* and *in vivo* studies are required to confirm our findings. In view of the encouraging results of the phase III clinical trials BLISS-LN [**18**] and AURORA 1 [**17**], treatment landscape in LN is changing, with combination treatment regimens challenging the sequential concept. Therefore, computational methods enabling systematic *in-silico* screening of combinatorial treatments in SLE merit further investigation.

In conclusion, our personalized molecular taxonomy strategy classified SLE patients into five molecular endotypes, based on their whole blood transcriptional disturbances. The stratified transcriptomes predicted patient endotype specific drug candidates, targeting the dysregulated gene expression profiles of the SLE patients. To propose novel, potentially beneficial agents, we designed an *in-silico*, signature-based drug repurposing pipeline. Considering the indispensable role of monocytes in the pathogenesis of SLE, we next employed two independent drug repurposing approaches, to identify novel compounds that might restore the transcriptional disturbances of these cells in SLE. Lastly, using a time-series, mouse kidney-specific transcriptome analysis, we constructed a clinical-transcriptome predictive model, that predicted patients that will develop LN.

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