HELLENIC REPUBLIC
National and Kapodistrian
University of Athens
EST. 1837

SCHOOL OF MEDICINE

1st Department of Propaedeutic and Internal Medicine

Director: Petros P. Sfikakis

«Η ΜΕΤΑ-ΜΕΤΑΓΡΑΦΙΚΗ ΕΠΕΞΕΡΓΑΣΙΑ ΤΟΥ RNA ΣΤΗ ΡΕΥΜΑΤΟΕΙΔΗ ΑΡΘΡΙΤΙΔΑ»

(“Adenosine-to-inosine RNA editing in rheumatoid arthritis”)

Nikolaos Vlachogiannis, MD

Doctoral dissertation

Athens, September 2020

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3. ΚΟΛΛΙΑΣ ΓΕΩΡΓΙΟΣ

4. ΣΤΕΛΛΟΣ ΚΩΝΣΤΑΝΤΙΝΟΣ

5. ΣΤΑΜΑΤΕΛΟΠΟΥΛΟΣ ΚΙΜΩΝ

6. ΤΕΚΤΟΝΙΔΟΥ ΜΑΡΙΑ

7. ΣΟΥΛΙΩΤΗΣ ΒΑΣΙΛΕΙΟΣ

Ευχαριστίες

Το ταξίδι μου στην έρευνα ξεκίνησε στο 2ο έτος της Ιατρικής, όταν σε ένα από τα εργαστήρια Φυσιολογίας ήρθα σε επαφή με την κυτταρική βιολογία του καρκίνου. Η έξαψη και το δέος που αισθάνθηκα με έκαναν να καταλάβω πως ήθελα να γίνω ερευνητής, να μην σταματήσω ποτέ να ρωτάω «γιατί» και να διοχετεύω την παιδική περιέργεια που διατηρώ σε δημιουργικές οδούς.

Μια σειρά ανθρώπων μου επέτρεψαν να ξεκινήσω αυτό το ταξίδι: ο Καθηγητής Φυσιολογίας κ. Μιχάλης Κουτσιλιέρης με δέχτηκε στο εργαστήριό του ως φοιτητή-ερευνητή και με βοήθησε να κάνω τα πρώτα μου ερευνητικά βήματα. Η Αν. Καθηγήτρια Κλειώ Μαυραγάνη με μύησε για πρώτη φορά στην έρευνα της Ανοσολογίας. Οι μεταδιδάκτορες Αδριανός Νέζος και Παναγιώτης Χριστόπουλος με μύησαν στην επιστημονική μέθοδο και την εργαστηριακή λεπτομέρεια. Στη συνέχεια, οι Καθηγητές Κώστας Στέλλος και Κίμων Σταματελόπουλος με βοήθησαν να βελτιωθώ περαιτέρω και να αποκτήσω διαφορετικές οπτικές γωνίες για το κάθε πρόβλημα, διευρύνοντας τις γνώσεις και τις δεξιότητές μου. Ο ερευνητής Βασίλης Σουλιώτης με τις επιστημονικές συζητήσεις μας δεν σταματά να με εμπνέει και να μου δημιουργεί την επιθυμία να δουλέψω πιο σκληρά. Τέλος, στο 6ο έτος της Ιατρικής γνώρισα τον μέντορά μου και επιβλέποντα της διατριβής αυτής, Καθηγητή Πέτρο Σφηκάκη, ο οποίος δεν σταμάτησε να με βοηθά να εξελιχθώ ως επιστήμονας αλλά και ως άνθρωπος.

Υπάρχει και μια σειρά ακόμη ανθρώπων που μέσα και έξω από το εργαστήριο με βοήθησαν να συνεχίσω. Η οικογένειά μου και οι φίλοι μου που με στήριζαν σε κάθε βήμα και απογοήτευση. Η Καθηγήτρια κυρία Τεκτονίδου και όλοι οι Ρευματολόγοι στην Α' Προπαιδευτική Παθολογική Κλινική που με βοήθησαν έμπρακτα στη στρατολόγηση

των ασθενών, αλλά και γενικότερα μοιράστηκαν τις γνώσεις τους μαζί μου. Το τεχνικό προσωπικό του εργαστηρίου και οι γραμματείς της κλινικής που με βοηθούσαν πάντα με χαμόγελο.

Σε όλους τους παραπάνω οφείλω ένα μεγάλο «ευχαριστώ»!

Ένα ξεχωριστό «ευχαριστώ» οφείλω στο Ίδρυμα Ωνάση που με υποστήριξε με υποτροφία για τις διδακτορικές μου σπουδές.

Τέλος, ευχαριστώ ξεχωριστά τον καθέναν από τους ασθενείς με ρευματοειδή αρθρίτιδα που συμμετείχαν στη μελέτη μου, οι οποίοι είναι η έμπνευσή μου και η πηγή δύναμης για να προχωρώ.

Ο όρκος του Ιπποκράτη (αρχαία ελληνική γλώσσα)

Ὄμνυμι Ἀπόλλωνα ἰητρὸν, καὶ Ἀσκληπιὸν, καὶ Ὑγείαν, καὶ Πανάκειαν, καὶ θεοὺς πάντας τε καὶ πάσας, ἵστορας ποιεύμενος, ἐπιτελέα ποιήσειν κατὰ δύναμιν καὶ κρίσιν ἐμὴν ὅρκον τόνδε καὶ ξυγγραφὴν τήνδε. Ἡγήσασθαι μὲν τὸν διδάξαντά με τὴν τέχνην ταύτην ἴσα γενέτῃσιν ἐμοῖσι, καὶ βίου κοινώσασθαι, καὶ χρεῶν χρηίζοντι μετάδοσιν ποιήσασθαι, καὶ γένος τὸ ἐξ ωὐτέου ἀδελφοῖς ἴσον ἐπικρινέειν ἄῤῥεσι, καὶ διδάξειν τὴν τέχνην ταύτην, ἢν χρηίζωσι μανθάνειν, ἄνευ μισθοῦ καὶ ξυγγραφῆς, παραγγελίης τε καὶ ἀκροήσιος καὶ τῆς λοιπῆς ἁπάσης μαθήσιος μετάδοσιν ποιήσασθαι υἱοῖσί τε ἐμοῖσι, καὶ τοῖσι τοῦ ἐμὲ διδάξαντος, καὶ μαθηταῖσι συγγεγραμμένοισί τε καὶ ὡρκισμένοις νόμῳ ἰητρικῷ, ἄλλῳ δὲ οὐδενί. Διαιτήμασί τε χρήσομαι ἐπ' ὠφελείῃ καμνόντων κατὰ δύναμιν καὶ κρίσιν ἐμὴν, ἐπὶ δηλήσει δὲ καὶ ἀδικίῃ εἴρξειν. Οὐ δώσω δὲ οὐδὲ φάρμακον οὐδενὶ αἰτηθεὶς θανάσιμον, οὐδὲ ὑφηγήσομαι ξυμβουλίην τοιήνδε. Ὁμοίως δὲ οὐδὲ γυναικὶ πεσσὸν φθόριον δώσω. Ἁγνῶς δὲ καὶ ὁσίως διατηρήσω βίον τὸν ἐμὸν καὶ τέχνην τὴν ἐμήν. Οὐ τεμέω δὲ οὐδὲ μὴν λιθιῶντας, ἐκχωρήσω δὲ ἐργάτῃσιν ἀνδράσι πρήξιος τῆσδε. Ἐς οἰκίας δὲ ὁκόσας ἂν ἐσίω, ἐσελεύσομαι ἐπ' ὠφελείῃ καμνόντων, ἐκτὸς ἐὼν πάσης ἀδικίης ἑκουσίης καὶ φθορίης, τῆς τε ἄλλης καὶ ἀφροδισίων ἔργων ἐπί τε γυναικείων σωμάτων καὶ ἀνδρῴων, ἐλευθέρων τε καὶ δούλων. Ἃ δ' ἂν ἐν θεραπείῃ ἢ ἴδω, ἢ ἀκούσω, ἢ καὶ ἄνευ θεραπηίης κατὰ βίον ἀνθρώπων, ἃ μὴ χρή ποτε ἐκλαλέεσθαι ἔξω, σιγήσομαι, ἄῤῥητα ἡγεύμενος εἶναι τὰ τοιαῦτα. Ὅρκον μὲν οὖν μοι τόνδε ἐπιτελέα ποιέοντι, καὶ μὴ ξυγχέοντι, εἴη ἐπαύρασθαι καὶ βίου καὶ τέχνης δοξαζομένῳ παρὰ πᾶσιν ἀνθρώποις ἐς τὸν αἰεὶ χρόνον. παραβαίνοντι δὲ καὶ ἐπιορκοῦντι, τἀναντία τουτέων.

Ο όρκος του Ιπποκράτη (απόδοση στη νέα ελληνική γλώσσα)

Ορκίζομαι στον Απόλλωνα τον ιατρό και στον Ασκληπιό και στην Υγεία και στην Πανάκεια και σε όλους τους/τις θεούς και επικαλούμαι τη μαρτυρία τους ότι θα υπηρετήσω κατά τη δύναμη και την κρίση μου τον όρκο αυτόν και το συμβόλαιο αυτό. Να θεωρώ τον δάσκαλό μου της τέχνης αυτής (Ιατρική) ίσο με τους γονείς μου και να μοιραστώ μαζί του το βιο μου και τα χρήματά μου. Να θεωρώ τους απογόνους του ίσους με του αδέλφια μου και να τους διδάξω αυτήν την τέχνη αν θέλουν να την μάθουν χωρίς δίδακτρα ή άλλο συμβόλαιο και να μεταδώσω τους κανόνες ηθικής και όλες τις άλλες ιατρικές μου γνώσεις στα παιδιά μου, στα παιδιά του δασκάλου μου και στους λοιπούς μαθητές που έχουν κάνει γραπτή συμφωνία μαζί μου και πήραν τον ιατρικό όρκο, αλλά σε κανέναν άλλο, να χρησιμοποιώ τη θεραπεία για να βοηθήσω τους πάσχοντες κατά τη δύναμη και κρίση μου, αλλά ποτέ για να τους βλάψω εκουσίως ή να τους αδικήσω. Να μην δώσω θανατηφόρο φάρμακο , ούτε αν κάποιος μου το ζητήσει, ούτε να δώσω ποτέ μια τέτοια συμβουλή. Παρομοίως, να μην δώσω ποτέ σε έγκυο μέσο που προκαλεί αποβολή. Να διατηρώ αγνή και καθαρή τη ζωή και την τέχνη μου και να μην χειρουργώ πάσχοντες από λιθίαση, αλλά να παραχωρώ την εργασία αυτή στους ειδικούς της τέχνης. Σε όσα σπίτια πηγαίνω, να μπω για να βοηθήσω τους ασθενείς αποφεύγοντας οποιαδήποτε εκούσια βλάβη, και κάθε γενετήσια πράξη με άνδρες ή γυναίκες, ελεύθερους και δούλους. Και οτιδήποτε βλέπω ή ακούω κατά την άσκηση των ιατρικών μου καθηκόντων ή και πέρα από τις επαγγελματικές μου ασχολίες στην καθημερινή μου ζωή, για τη ζωή των ανθρώπων που δεν πρέπει να κοινοποιηθούν, να σιωπήσω και να διαφυλάξω τα μυστικά. Αν τηρώ τον όρκο αυτό πιστά και δεν τον παραβώ, είθε να χαίρω για πάντα της εκτίμησης των ανθρώπων για τη ζωή και για την τέχνη μου, αν όμως τον παραβώ και επιορκήσω, ας υποστώ τα αντίθετα.

The Hippocratic oath

I swear by Apollo Physician, by Asclepius, by Hygeia, by Panacea, and by all the Gods and Goddesses, making them my witnesses, that I will carry out, to the best of my ability and judgment, this oath and this contract of honour. To hold my teacher of this art (Medicine) equal to my own parents; to share with them (my teacher) my life and when they are in need of money to share mine with them; to consider their family equal to my siblings, and to teach them this art (Medicine), if they want to learn it, without fee or any other contract; to impart precept, oral instructions, and all my knowledge to my own children, my teacher's children, and to indentured pupils who have taken the physician's oath, but to nobody else. I will use treatment to help the sick to the best of ability and judgment, but never to injure or perform any wrong-doing. I awill never administer a poison to cause anybody's death even when asked to do so, nor will I ever suggest such a course. I will never give to a woman a pessary to cause abortion. I will keep pure and honourable both my life and my art (Medicine). I will not perform surgery, not even on sufferers from lithiasis, but I will leave this to the specialists. In any homes that I enter, I will enter to help the sick, and I will abstain from all intentional wrong-doing or harm, and I will never engage in any intercourse with men or women, slaves or free. Whatever I shall see or hear in the course of my profession, as well as outside of my professional life, if it should not be published, I will never share it with anyone, holding it as a holy secret. If I carry out this oath, and never break it, may I enjoy forever respect and reputation among all people for my life and for my art; but if I break this oath and forswear myself, may the opposite befall me.

Curriculum Vitae

Biographical Sketch

Name:	Nikolaos I. Vlachogiannis
Work address:	Ag. Thoma 17, 11527, Athens, Greece
Tel. / e-mail:	+306974589646 / nivlachogiannis@gmail.com
Date of birth:	13/09/1992

Education

10/2016 - 09/2020	**Doctoral studies:** "Adenosine-to-inosine RNA editing in rheumatoid arthritis" Immunology Lab, Rheumatology Unit, 1st Dept. of Propaedeutic & Internal Medicine, National and Kapodistrian University of Athens, Athens, Greece Supervisor: Prof. P.P. Sfikakis
2010-2016	**Medical Degree**: School of Medicine, National and Kapodistrian University of Athens, Athens, Greece (Grade: 8.67/10, Summa Cum Laude) *During my studies I also worked as undergraduate researcher at the Department of Experimental Physiology (Chair: Prof. Michael Koutsilieris, M.D., Ph.D)*

Scholarships and awards

06/2019	**EULAR travel bursary**
12/2018	**3rd best basic/translational research award in the 26th Greek Rheumatology Congress** Abstract: "DNA damage accumulation, chromatin deregulation and defective DNA repair in patients with systemic autoimmune diseases" (equal 1st author)
06/2018	**Lindau Nobel Laureaute Meeting** Partcipation in the 68th Lindau Nobel Laureate Meeting
10/2017	**Alexander S. Onassis Public Benefit Foundation Scholarship** for doctoral studies at National and Kapodistrian University of Athens
06/2017	**EULAR travel bursary**
9/2013 – 2/2014	**ERASMUS programme,** Goethe University Frankfurt am Main, Germany
2010- 2016	**Undergraduate scholarship** "Papadakis scholarship of excellence" for undergraduate students of the National and Kapodistrian University of Athens

Publications in peer-reviewed journals

(Google scholar, 09/2020: total citations: 168, h-index:6)

Original articles:

1) **Vlachogiannis N.I.**, Gatsiou A., Silvestris D.A., Stamatelopoulos K., Tektonidou M.G., Gallo A., Sfikakis P.P.*, Stellos K.*, Increased adenosine-to-inosine RNA editing in rheumatoid arthritis, *J. Autoimmun*. 106 (2020) 102329. *equal contribution **IF: 7.54**
2) Liberale L, Akhmedov A., **Vlachogiannis N.I.** et al., Sirtuin 5 promotes arterial thrombosis by blunting the fibrinolytic system, Cardiovasc Res 2020 (in press). **IF: 8.168**
3) Tektonidou M.G., Kravvariti E., Vlachogiannis N.I., Georgiopoulos G., Mantzou A., Sfikakis P.P., Stellos K.*, Stamatelopoulos K.* Clinical value of amyloid-beta1-40 as a marker of thrombo-inflammation in Antiphospholipid Syndrome, Rheumatology (Oxford)- [in press] **IF: 5.606**
4) Mareti A., Kritsioti C., Georgiopoulos G., **Vlachogiannis N.I.**, Delialis D., Sachse M., Sopova K., Koutsoukis A., Kontogiannis C., Patras R., Tual-Chalot S., Koureas A., Gatsiou A., Stellos K., Stamatelopoulos K., Cathepsin B expression is associated with arterial stiffening and atherosclerotic vascular disease, *Eur J Prev Cardiol*. (2019) 2047487319893042. **IF: 5.64**
5) Souliotis VL, **Vlachogiannis NI**, Pappa M, Argyriou A, Sfikakis PP. DNA damage accumulation, defective chromatin organization and deficient DNA repair capacity in patients with rheumatoid arthritis. *Clin Immunol. 2019 Jun;203:28-36* **IF:3.56**
6) **Vlachogiannis NI**, Nezos A, Tzioufas AG, Koutsilieris M, Moutsopoulos HM, Mavragani CP. Increased frequency of the PTPN22W* variant in primary Sjogren's Syndrome: Association with low type I IFN scores. *Clin Immunol.* 2016; 173:157-160. **IF:3.99**

Review articles-Editorials:

7) **Vlachogiannis N.I.**, Stellos K. Circulating Progenitor Cells Predict Clinical Outcomes in Patients With Coronary Artery Disease and Renal Insufficiency, JACC Basic Transl Sci. 2020;5(8):783-785.
8) Lazaridis C., Vlachogiannis N.I., Bakogiannis C., Spyridopoulos I., Stamatelopoulos K., Kanakakis I., Vassilikos V., Stellos K., Involvement of cardiovascular system as the critical point in coronavirus disease 2019 (COVID-19) prognosis and recovery, Hell. J. Cardiol. (2020): S1109-9666(20)30093-2 **IF: 4.047**
9) Stakos D.A., Stamatelopoulos K., Bampatsias D., Sachse M., Zormpas E., **Vlachogiannis N.I.**, Tual-Chalot S., Stellos K., The Alzheimer's Disease Amyloid-Beta Hypothesis in Cardiovascular Aging and Disease: JACC Focus Seminar, *J. Am. Coll. Cardiol*. 75 (2020) 952–967. **IF:18.64**
10) Souliotis V.L.*, **Vlachogiannis N.I.***, Pappa M., Argyriou A., Ntouros P.A., Sfikakis P.P., DNA Damage Response and Oxidative Stress in Systemic Autoimmunity, *Int J Mol Sci*. 21 (2019). *equal contribution **IF:4.18**

11) Gatsiou A, **Vlachogiannis N**, Lunella FF, Sachse M, Stellos K. Adenosine-to-Inosine RNA Editing in Health and Disease. *Antioxid. Redox Signal.* 2018; 29(9):846-863 **IF:6.53**
12) Christopoulos PF*, **Vlachogiannis NI***, Vogkou CT, Koutsilieris M, The Role of the Androgen Receptor Signaling in Breast Malignancies. *Anticancer Res.* 2017; 37(12):6533-6540. *equal contribution **IF:1.87**
13) Sfikakis PP, **Vlachogiannis NI,** Christopoulos PF. Cadherin-11 as a therapeutic target in chronic, inflammatory rheumatic diseases, *Clin Immunol.* 2017;176:107-113. **IF:3.99**
14) Vogkou CT*, **Vlachogiannis NI,*** Palaiodimos L, Kousoulis AA. The causative agents in Infective Endocarditis: a systematic review comprising 33,214 cases. *Eur J Clin Microbiol Infect Dis.* 2016; 35(8):1227-45. *equal contribution **IF:2.54**
15) Gkioka E, Msaouel P, Philippou A, **Vlachogiannis NI**, Vogkou CT, Margiolis A, Koutsilieris M. Review: The Role of Insulin-like Growth Factor-1 Signaling Pathways in Uterine Leiomyoma. *Vivo Athens Greece 29, 637–649.* **IF: 0.95**

Presentations in Biomedical Congresses (oral/poster presentations)

12-15/6/2019	2019 European League against Rheumatism Congress Madrid, Spain
24-29/03/2019	Next-Generation Epitranscriptomics in Health and Disease
23-24/03/2019	RNA and DNA Editing and Modification: Mechanism, Function and Tools for Precision Medicine
6-9/12/2018	26th Congress of the Hellenic Society of Rheumatology, Athens, Greece
3-6/10/2018	EMBL Symposium: “The Complex Life of RNA”
12-14/4/2018	XVII Mediterranean Congress of Rheumatology
14-17/6/2017	2017 European League against Rheumatism Congress Madrid, Spain
24-27/3/2016	6th Turkish Greek Rheumatology Days, Istanbul, Turkey
8-11/12/2016	25th Congress of the Hellenic Society of Rheumatology, Athens, Greece
15-17/5/2015	21st Scientific Congress of Greek Medical Students, Athens, Greece
4-6/5/2012	18th Scientific Congress of Greek Medical Students, Athens, Greece
6-8/5/2011	17th Scientific Congress of Greek Medical Students, Crete, Greece

Language skills

English: Fluent (C2-Michigan University), IELTS (11/2018): 8.0/9.0
German: Very Good (C1-Goethe Institut)

Scientific bodies

Member of: Emerging EULAR Network (EMEUNET)

Table of contents

List of abbreviations

Abbreviation	Explanation
RA	rheumatoid arthritis
A-to-I	adenosine-to-inosine
dsRNA	double-stranded RNA
ADAR	adenosine deaminase acting on RNA
ADAR1p150	long 150kDa ADAR1 isoform
ADAR1p110	short 110kDa ADAR1 isoform
GluRB	Glutamate receptor subunit B
miRNA	microRNAs
circRNA	circular RNAs
lncRNA	long-non-coding RNAs
IFN	interferon
ISGs	Interferon-stimulated genes
PRRs	pattern-recognition receptors
TLRs	Toll-like receptors
RIG-I	retinoic acid inducible gene I
MDA5	melanoma differentiation-associated protein-5
MAVS	Mitochondrial Anti-Viral Signaling
IRF	interferon responsive factors
NF-kB	nuclear factor kappa b
MEFs	mouse embryonic fibroblasts
AZIN1	antizyme inhibitor 1
HCC	hepatocellular carcinoma

GLI1	Glioma associated oncogene 1
CVD	cardiovascular disease
CTSS	cathepsin S
HuR	Human Antigen R
ROS	Reactive oxygen species
HDV	Hepatitis Delta Virus
HIV	Human Immunodeficiency Virus
SLE	Systemic Lupus Erythematosus
AGS	Aicardi-Goutières syndrome
BSN	Bilateral striatal necrosis
RA	Rheumatoid Arthritis
bDMARDs	biologic disease-modifying anti-rheumatic drugs
RF	rheumatoid factor
anti-CCP	anti-cyclic citrullinated peptide
PBMCs	peripheral blood mononuclear cells
csDMARDs	conventional synthetic disease-modifying antirheumatic drugs

Summary

Adenosine-to-inosine (A-to-I) RNA editing of Alu retroelements is a primate-specific mechanism mediated by adenosine deaminases acting on RNA (ADARs) that diversifies transcriptome by modifying selected nucleotides in RNA molecules. Herein, we tested the hypothesis that A-to-I RNA editing is altered in rheumatoid arthritis (RA). Synovial expression of ADAR1 and its isoforms, ADAR1p110 and ADAR1p150, was analysed in 152 RA patients and 50 controls. Peripheral blood mononuclear cells (PBMCs) derived from 14 healthy subjects and 19 patients with active RA at baseline and after 12-week treatment were examined for ADAR1p150 and ADAR1p110 isoform expression by RT-qPCR. RNA editing activity was analysed by AluSx$^+$ Sanger-sequencing of cathepsin S, an extracellular matrix degradation enzyme also involved in antigen presentation. ADAR1 was significantly over-expressed in RA synovium regardless of disease duration. Similarly, ADAR1p150 isoform expression was significantly increased in the synovium and blood of active RA patients, while ADAR1p110 levels were similar between patients and controls. Individual nucleotide analysis revealed that A-to-I RNA editing rate in cathepsin S AluSx$^+$ was also significantly increased in RA patients. Both baseline ADAR1p150 expression and individual adenosine RNA editing rates in cathepsin S AluSx$^+$ decreased after treatment only in those patients with good clinical response. Upregulation of the expression and/or activity of the RNA editing machinery was associated with a higher expression of edited Alu-enriched genes including cathepsin S and TNF receptor-associated factors 1,2,3 and 5. In conclusion, herein we describe a previously unrecognized regulation and role of ADAR1p150-mediated A-to-I RNA editing in post-transcriptional control of gene expression in RA, which underpins therapeutic response and fuels inflammatory gene expression, thus representing an interesting therapeutic target.

Περίληψη (Summary in Greek)

Η μετα-μεταγραφική επεξεργασία του RNA (RNA editing), με πιο συχνή μορφή τη μετατροπή της αδενοσίνης σε ινοσίνη (adenosine-to-inosine RNA editing), είναι μια βασική βιολογική διεργασία η οποία λαμβάνει χώρα κυρίως στις Alu περιοχές του μεταγραφώματος και διαμεσολαβείται από τα ένζυμα ADAR1 και ADAR2. Το ADAR1 έχει 2 ισομορφές, μια πιο μακριά (ADAR1p150) και μια βραχεία (ADAR1p110). Η σημασία του μηχανισμού αυτού τονίζεται από τον ενδομήτριο θάνατο των ποντικιών τα οποία στερούνται της ενζυματικής δράσης του ADAR1. Στην παρούσα μελέτη εξετάσαμε τη συμμετοχή του RNA editing στη Ρευματοειδή Αρθρίτιδα (PA), μια χρόνια φλεγμονώδη (αυτοάνοση) νόσο που επηρεάζει περίπου το 1% του πληθυσμού. Πιο συγκεκριμένα εξετάσαμε το ρόλο του RNA editing στη ρύθμιση των επιπέδων φλεγμονωδών μορίων. Για το σκοπό αυτό αναλύσαμε ένα RNA sequencing dataset που περιλάμβανε συνολικά 202 βιοψίες αρθρικού υμένα [28 υγιείς, 22 ασθενείς με οστεοαρθρίτιδα, 57 ασθενείς με πρώιμη PA (διάρκεια νόσου<1 έτος) και 95 ασθενείς με εγκατεστημένη PA]. Επίσης, συλλέξαμε αίμα από ασθενείς με ενεργό PA πριν- και 3 μήνες μετά την έναρξη νέας θεραπείας και απομονώσαμε λεμφομονοπύρηνα κύτταρα (PBMCs). Οι τεχνικές που χρησιμοποιήθηκαν ήταν ποσοτική αλυσιδωτή αντίδραση πολυμεράσης (qPCR) και Sanger sequencing. Τα αποτελέσματα της μελέτης ανέδειξαν σημαντική αύξηση της ισομορφής ADAR1p150 τόσο στον αρθρικό υμένα όσο και στο αίμα των ασθενών, ενώ τα επίπεδα της ισομορφής ADAR1p110 καθώς και του ενζύμου ADAR2 δε διέφεραν μεταξύ ασθενών και υγιών. Στη συνέχεια, εξετάσαμε το RNA editing της cathepsin S, ενός μορίου που εμπλέκεται ενεργά στην αντιγονοπαρουσίαση και στην διάσπαση εξωκυττάριας ουσίας και που έχει προταθεί ως σημαντικό κομμάτι της παθοφυσιολογίας της PA ή ακόμη και ως θεραπευτικός στόχος. Παρατηρήσαμε ότι το RNA editing στο στοιχείο $AluSx^+$ της cathepsin S ήταν σημαντικά αυξημένο στα λεμφομονοπύρηνα κύτταρα ασθενών με ενεργό PA. Με ιδιαίτερο ενδιαφέρον παρατηρήσαμε πως τόσο η έκφραση του μορίου ADAR1p150 όσο και το RNA editing του $AluSx^+$ της cathepsin S μειώθηκαν σημαντικά μετά τη θεραπεία στους ασθενείς που είχαν καλή ανταπόκριση στη φαρμακευτική αγωγή (κριτήρια EULAR), ενώ παρέμειναν αμετάβλητα στους ασθενείς με μέτρια ή καθόλου ανταπόκριση. Τέλος, η έκφραση της cathepsin S, που ήταν αυξημένη τόσο στον αρθρικό υμένα όσο και στα λεμφομονοπύρηνα κύτταρα των ασθενών, συσχετιζόταν ισχυρά τόσο με το ADAR1p150 όσο και με τα επίπεδα του RNA editing. Συμπερασματικά, προτείνουμε πως το RNA editing μπορεί να δρα ως ένα επιπρόσθετο, μετα-μεταγραφικό επίπεδο ρύθμισης της γονιδιακής έκφρασης στη PA.

THEORETICAL BACKGROUND

(ΓΕΝΙΚΟ ΜΕΡΟΣ)

Introduction

In the 21st century rheumatoid arthritis (**RA**) remains an incurable disease affecting approximately 0.5-1% of the population in Western world. [1] Despite significant scientific advances in the past decades, such as the development of monoclonal antibodies targeting central components of the auto-immune inflammatory milieu (TNF, IL6, IL-1), the percentage of patients who achieve disease remission remains unsatisfactorily low, [2] while life expectancy of patients with RA is significantly shortened. In the ongoing quest to better understand the pathophysiology of RA with the utter goal to develop biomarkers for disease progression and drug response and a “tailored” precision medicine approach, as well as to identify novel therapeutic targets, recent technological advancements have contributed significantly to the exploration of previously unknown mechanisms, such as RNA modifications.

RNA, historically considered as a passive intermediate between DNA and proteins, is increasingly recognized as rather a multi-functional, regulatory molecule dictating the fate of the proteome. The current thesis focuses on the role of adenosine-to-inosine RNA editing, the most common RNA modification, in RA and its role in regulation of proinflammatory gene expression.

1. Adenosine-to-inosine RNA editing

1.1 RNA modifications: expanding the RNA alphabet from 4 to >170 nucleotides

According to the central dogma of biology, as it was originally described by Francis Crick more than 50 years ago, [3,4] the information of the genetic code (DNA-genome) is transcribed into a messenger molecule (RNA-transcriptome) in order to be finally translated into a protein (proteome) with certain 3D-structure and function. For many years RNA has been thought as a passive intermediate carrier of genetic information. However, recent research advances suggest that RNA metabolism actively regulates protein expression and function. [5] Of interest, RNA-binding proteins comprise more than 10% of the proteome being highly conserved from archaea to higher eukaryotes, suggestive of their important role in evolution. [6]

To add to the complexity of the transfer of genetic information, DNA is subjected to various modifications (i.e. methylation), which are collectively termed the epigenome. Similarly, RNA is subjected to numerous modifications (as this text is being written more than 170 RNA modifications have been described), which expand the RNA alphabet from 4 (A, U, C, G) to hundreds of bases, controlling RNA metabolism and in consequence protein expression and function. [7] Recent technological advances, such as the development of 2nd generation RNA-sequencing or long read sequencing technologies (i.e. Oxford Nanopore sequencing), have enabled the detection and quantification of RNA modifications allowing researchers to explore the “epitranscriptome” and its role from physiology to disease (**Figure 1**). [8]

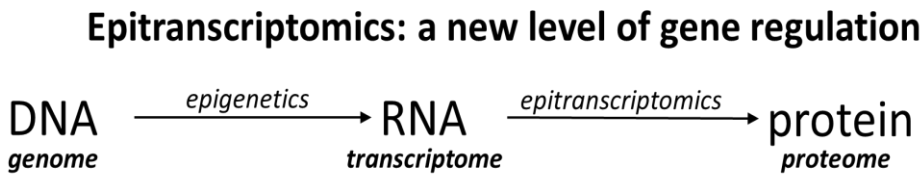

Figure 1: Schematic representation of the central dogma of biology showing the new field of "epitranscriptomics".

RNA modifications can be roughly divided into 2 main categories: substitutional and non-substitutional, depending on whether they lead to a nucleotide sequence change. Some of the most prevalent non-substitutional RNA modifications include N6-methyladenosine (m6A), 5-methylcytosine (m5C) and pseudo-uridylation. On the other hand, the most common substitutional RNA modification is adenosine-to-inosine RNA editing. [9] While non-substitutional RNA modifications such as m6A RNA methylation require chemical modification or specific antibodies to be detected, A-to-I RNA editing is readily detectable by sequencing. [10]

1.2 Alu RNA editing: a primate-specific, post-transcriptional mechanism regulating RNA metabolism

Among the numerous RNA modifications, adenosine-to-inosine (**A-to-I**) RNA editing is the most common substitutional modification. [9] A-to-I RNA editing was initially described in *Xenopus laevis* oocytes and embryos as the unwinding of double-stranded RNA (**dsRNA**) by some unknown enzymes, [11] which shortly thereafter were identified as the adenosine deaminase acting on RNA (**ADAR**) family of enzymes. [12,13]

Adenosine Deaminase Acting on RNA (ADAR) family of enzymes

The ADAR family of enzymes consists of 3 members, namely ADAR1, ADAR2 and ADAR3. All three members of the ADAR family share common domains, such as double-stranded RNA binding domain (dsRBD), a nuclear localization domain (NLS) and a deaminase domain (**Figure 2**). However, to date only ADAR1 and ADAR2 have been shown to induce A-to-I RNA editing, while ADAR3 is considered to be catalytically inactive *in vivo*. [14] Of note, ADAR1 comes in two isoforms, a larger 150kDa isoform (**ADAR1p150**) that originates from an alternative interferon-inducible promoter and a shorter 110kDa isoform (**ADAR1p110**). [15,16] ADAR1p110 mainly resides in the nucleus and is more abundant than ADAR1p150 under physiological conditions. [17] On the other hand, ADAR1p150 has a unique nuclear export signaling (NES) domain and therefore is able to shuttle between the nucleus and the cytoplasm. [17,18] The potential of ADAR1p150 to localize in the cytoplasm has multiple physiologic and pathophysiologic extensions, which I will analyze in the next chapters.

Figure 2

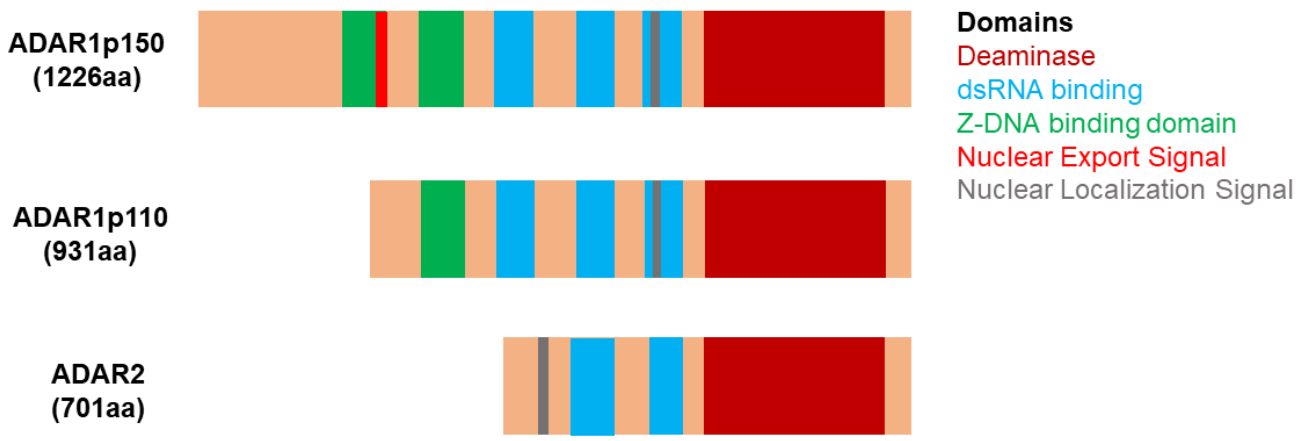

Figure 2. The adenosine deaminase acting on RNA (ADAR) family of enzymes. *ADAR family consists of three members: two ADAR1 isoforms, the longer ADAR1p150 (150kDa; 1226aa) and the shorter ADAR1p110 (110kDa; 931aa), ADAR2 (701aa) and the catalytically inactive ADAR3 (739aa- not shown). Z- DNA binding domain(s) present in ADAR1p150 and ADAR1p110, but not in ADAR2, are depicted in green. Double stranded RNA (dsRNA) binding domain (light blue), deaminase domain (editase- dark red) and nuclear localization signal (NLS- grey) are present in both ADAR1 isoforms and ADAR2. On the other hand, ADAR1p150 harbors a nuclear exportation signal (NES; bright red) accounting for its cytoplasmic localization.*

Alu elements

As already mentioned, both ADAR1 and ADAR2 possess a dsRNA-binding domain which allows them to bind to RNA duplexes and edit the adenosine residues. [19,20] Therefore, the most common site where RNA editing takes place are Alu elements. Alu elements are endogenous retrotransposons, approximately 200-300 nucleotides in length, that belong to the family of SINEs (short interspersed nuclear elements). Of note, Alu elements comprise approximately 10% of the human genome and they have highly similar sequences, which allows them to form local RNA duplexes, which are excellent substrates for RNA editing. [21,22]

Alu editability

There are several factors that affect the editability of Alu elements. Eisenberg and colleagues analysed two large RNA-seq. datasets consisting of RNA samples derived from 16 human tissues and were able to determine 5 different parameters that may account for Alu editability: [23]

a) the distance to the nearest reversely oriented Alu, which accounts for approximately 30% of Alu editability.

b) the number of reversely oriented repeats in the vicinity of the Alu, which led to increased Alu editability.

c) the number of same-strand repeats in close proximity with the Alu, which negatively affected the editability

d) the length of the Alus

e) the consensus strand of the Alu repeats is more strongly edited than the reverse strand.

1.3 The role of RNA editing in physiology and innate immune response

The effect of RNA editing on RNA metabolism

A-to-I RNA editing consists of the hydrolytic deamination of adenosine at C-6 converting it into inosine (hence a substitutional RNA modification; **Figure 3**). Inosine is in turn recognized by the cellular machinery as guanosine with multiple consequences for the fate of the RNA molecules depending, among others, on the site of RNA editing events. [14]

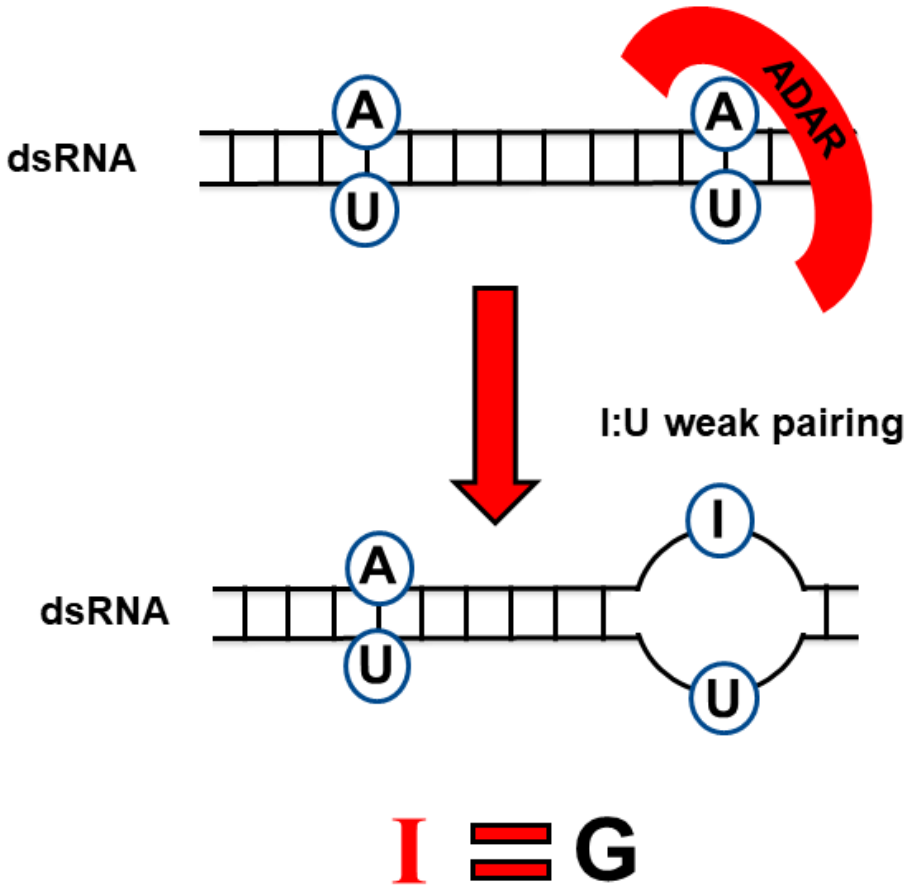

Figure 3. *ADARs bind to double stranded RNAs (dsRNAs) and deaminate adenosine residues turning them into inosines. Inosine-uracil pairs form weak bonds and thus dsRNA unwinds locally to single-stranded RNA (ssRNA). Inosine shares chemical properties with guanosine, therefore being further recognized as guanosine by the cellular machineries.*

In the rare event that A-to-I RNA editing takes place in the coding sequence of mRNA it can lead to recoding events. One of the best described RNA editing events in coding regions takes place in Glutamate receptor subunit B (GluRB) precursor messenger RNA (pre-mRNA) (Gria2) of the aminomethylphosphonic acid (AMPA) receptor family, with biological consequences for the ion flow in glutamate-gated channels. [24] Two edited positions within the GluRB pre-mRNA, edited exclusively by the second RNA editor, ADAR2, lead to recoding events in exon 11 (CAG: Q/CI/GG: R) and exon 13 (CGA:R/GGI/G:G). Of interest, the first recoding event, which is almost 100% edited, is indispensable for proper function of GluRB and its absence leads to post-natal lethality of mice. [25]

However, the vast majority of RNA editing events take place in non-coding sites affecting various aspects on RNA metabolism such as splicing, microRNA (**miRNA**) targeting, biogenesis of circular RNAs (**circRNAs**), long non-coding RNA (**lncRNA**) re-targeting and finally mRNA stability (**Figure 4**). [14,19] Interestingly, regulatory mechanisms have been mapped within the “dark matter” (the non-coding genome), which may account for the complexity in higher primates [26] and be crucially involved in several human pathologies. Thus, intensive efforts have been focused on unravelling the molecular role of A-to-I RNA editing in dark matter derivatives such as miRNAs, circRNAs, lncRNAs and Alu repetitive elements.

Figure 4

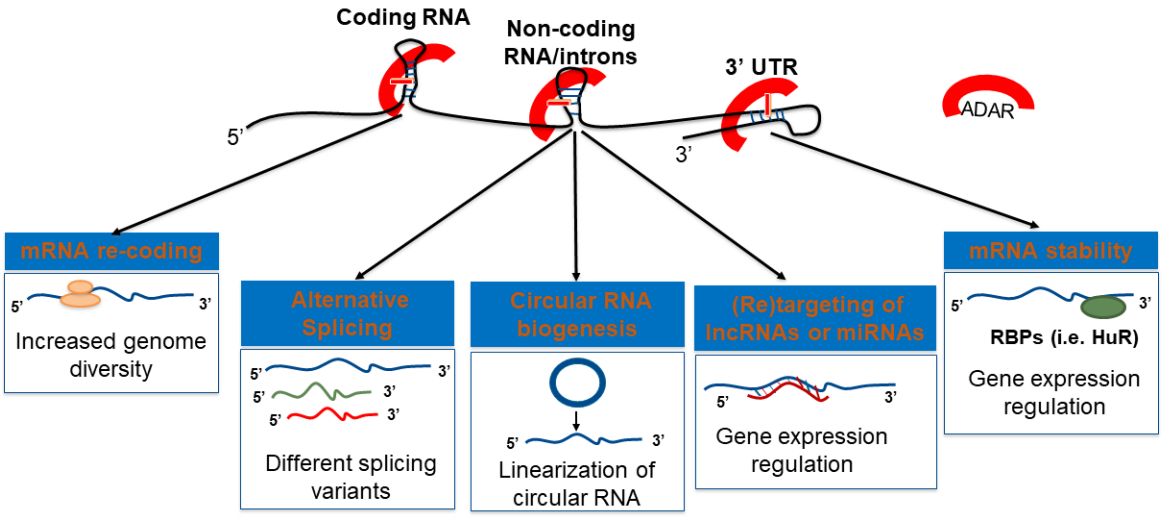

Figure 4. Main molecular consequences of ADAR-induced RNA editing.

ADAR1- or ADAR2 (ADAR1/2) -induced RNA editing in coding RNA may result in mRNA recoding and thus increase the genome diversity. If editing occurs in introns, it may give rise to alternative transcripts which may or may not translate into protein, probably with altered function. Moreover, RNA editing may affect circular RNA (circRNA) biogenesis. A-to-I RNA editing of Alu regions surrounding circRNA inhibits the biogenesis of circRNAs. A-to-I RNA editing in the seed region of miRNAs leads to re-targeting events with functional consequences. Notably, long non coding RNA (lncRNA) binding to pre-mRNA can create a double-stranded RNA (dsRNA), a prerequisite for ADAR1/2-mediated RNA editing. ADAR1 editing of inverted Alu present in 3'UTR of a transcript may result in unwinding und subsequent recruitment of HuR. HuR binding stabilizes transcripts and ensures the proper processing into protein.

A-to-I RNA editing is essential for life

ADAR1 is indispensable for life, since its genetic ablation results in *in utero* lethality of mouse embryos by embryonic day 12.5 (E12.5) associated with liver degeneration and anemia. [27–29] Histological analysis of these embryos revealed widespread apoptosis, fetal liver disintegration and ineffective hematopoiesis, specifically with regards to erythroid and myeloid/granulomatous progenitors [27,28]. Taken together, these findings suggested an essential role of ADAR1 in hematopoiesis, organ homeostasis and development.

Given the binary nature of ADAR1, as an RNA-binding protein and as an RNA editing enzyme, later studies aimed to dissect the functional role of this enzyme *in vivo*. Using an elegant experimental approach, Liddicoat *et al.* generated mice bearing a constitutive knock-in of an RNA editing-deficient ADAR1 mutant (**Adar1$^{E861A/E861A}$**). Of note, these deficient mice also died *in utero* around E13.5 displaying a similar phenotype (liver failure, defective hematopoiesis) and a prominent activation of innate immune response [30] recapitulating in principle the initially observed phenotype of ADAR1 KO mice. [27,28,31] These findings underline that ADAR1-induced RNA editing is essential for homeostasis and life itself.

Gene expression profiling of *Adar1*$^{-/-}$ embryos, as well as *Adar1*$^{-/-}$ fetal liver, showed the aberrant expression of interferon (**IFN**) stimulated genes (**ISGs**). [27] Furthermore, *in vitro* studies on hematopoietic stem cells and hematopoietic progenitor cells revealed the induction of an aberrant innate immune response in absence of ADAR1 suggesting a role for ADAR1 as a repressor of the IFN signaling. [31,32]

The role of A-to-I RNA editing in innate immune response

In eukaryotic cells pattern-recognition receptors (**PRRs**), including Toll-like receptors (**TLRs**) and other cytoplasmic receptors trigger innate immune response and inflammation to overcome viral infection. [33] DsRNA may be recognized by TLR-3, which is mainly located at the cell surface and at the endosome, or by cytoplasmic receptors belonging to the retinoic acid inducible gene I (**RIG-I**) like receptors (**RLRs**) family, mainly RIG-I and melanoma differentiation-associated protein-5 (**MDA5**). [34]

An initial *in vitro* observation that oligonucleotides containing inosine-uracil (I:U) base pairs (hallmarks of RNA editing events) suppress expression of interferon-stimulated genes by binding to MDA5 or RIG-I, [35] suggested that ADAR1 and RNA editing may play a primary role in preventing sensing of endogenous dsRNA as "non-self" by the innate immune system. In contrast, other researchers proposed that ADAR1-mediated suppression of innate immune response depends on its ability to bind dsRNA and physically contact RIG-I rather than on its catalytic activity. [36]

In order to rescue the embryonic *Adar1*$^{-/-}$ lethal phenotype and gain further insights on the underlying molecular mechanisms, researchers generated a double mutant mouse for Adar1 and Ifnar1 (the common IFN-α and IFN-β receptor) or Stat1 (a key mediator of systemic IFN responses). [37] In this way, the survival of *Adar1*$^{-/-}$*/Ifnar1*$^{-/-}$ or *Adar1*$^{-/-}$*/Stat1*$^{-/-}$ embryos was extended by 2-4 days compared to *Adar1*$^{-/-}$ embryos, however the embryos did not make it to birth showing similar deficiencies to those observed in *Adar1*$^{-/-}$. [37]

These results suggested that type I IFN is not the sole cytokine responsible for the lethal phenotype of *Adar1*$^{-/-}$ mice. Thus, in order to potentially achieve a rescue of

the *Adar1*$^{-/-}$ lethality researchers turned to blocking a more upstream mediator of the dsRNA-induced innate immune response. Mitochondrial Anti-Viral Signaling (**MAVS**) protein is an adaptor protein that resides at the mitochondrial membrane and, when activated, triggers in turn the activation of the transcription factors IRF3/IRF7 (interferon responsive factors) and NF-kB (nuclear factor kappa b) to induce the transcription of type I IFNs and proinflammatory cytokines. MAVS is activated upon recognition of viral ("non-self") dsRNA by either RIG-I or MDA5**.** [38–42] Of interest, *Adar1*$^{-/-}$*/Mavs*$^{-/-}$ double mutant embryos survived up to birth, but only to die a day or maximum ten days later, [37] with the majority of the neonates dying within the first two days. [43] In the first study, histological analysis revealed apparently normal morphology of liver, heart and other organs, but blood analysis indicated persistently elevated IFN and IL-1 levels. [37] In the second study, severe defects in kidneys' architecture, disturbed intestinal homeostasis, lack of organization in lymphoid follicles in both lymph nodes and spleen, and defects in B-cell maturation were present in double knockout mutant mice. [43] These findings suggested an MAVS-independent role of ADAR1 in hematopoiesis and an essential role of ADAR1 in multiple organs' development and homeostasis. [37,43]

The overall observations supported the notion that knocking out an upstream dsRNA sensor, RIG-I or MDA5, might confer greater protection to aberrant innate immune response in the absence of ADAR1. Surprisingly, in the same study, *Adar1*$^{-/-}$*/Mda5*$^{-/-}$ double KO mutants displayed a similar survival curve as the *Adar1*$^{-/-}$*/Mavs*$^{-/-}$ mice and died postnatally within a week. [43] However, when *Mda5*$^{-/-}$ mice were crossed with *Adar1*$^{E861A/E861A}$ (editing deficient knock-in mutation), *Adar1*$^{E861A/E861A}$*/Mda5*$^{-/-}$ mutants not only survived past weaning but also displayed no abnormalities, apart from slightly smaller size compared with littermate controls. [30]

These breakthrough findings provided the basis for MDA5 to be considered as the primary sensor of endogenous unedited dsRNAs.

In parallel, a key question remained regarding which ADAR1 isoform contributes the most to the lethal phenotype. As previously mentioned, two isoforms give rise to two ADAR1 proteins with distinct molecular weights, ADAR1p150 (long isoform, 150kDa) and ADAR1p110 (short isoform, 110kDa). The long isoform is induced by an interferon-responsive promoter utilizing a translational start site in exon 1A, while the shorter isoform is constitutively expressed using a promoter present in exon 1B or 1C and an alternative translational start site residing in exon 2. [15]

In a first attempt to clarify this point, Ward *et al.* generated mice lacking the ADAR1p150 isoform by exclusively removing exon 1A, thereby preserving the ADAR1p110-specific exon 1B intact. Interestingly, these mice displayed the same phenotype observed in the double Adar1-knockout mice (embryonic death at E11-12) [27] suggesting that the interferon-induced ADAR1p150 isoform may account for the lethal phenotype. [44] Some concerns with respect to the applied genetic strategy were raised, questioning whether ADAR1p110 protein levels are affected in the ADAR1p150$^{-/-}$ mice, participating in the phenotype. [45] The answer to this issue came a few years later by Pestal *et al.* who used the same *Adar1p150*$^{-/-}$ strain and demonstrated that ADAR1p110 protein levels were unaffected in isolated mouse embryonic fibroblasts (**MEFs**). These results corroborated the crucial role of ADAR1p150 in the lethal phenotype.

As a next step, researchers examined the effects of *Adar1p150*$^{-/-}$*/Mavs*$^{-/-}$ double knockout [43]. Of note, these mice survived to weaning with no apparent abnormalities, albeit smaller in size than their littermate controls, in line with the observations in *Adar1*$^{E861A/E861A}$*/Mda5*$^{-/-}$ mice. [30] Detailed histological examination

suggested that the morphological deficiencies present in the kidneys of *Adar*$^{-/-}$*/Mavs*$^{-/-}$ mice were no longer manifested in *Adar1p150*$^{-/-}$*/Mavs*$^{-/-}$ mice. Moreover, A-to-I RNA editing analysis of an exemplary ADAR1-edited target (5-HT2C receptor) showed no decrease in RNA editing levels, indicating that the residual ADAR1p110 expression and editing activity in these mice were sufficient for the recovery of kidney development. [43] On the other hand, intestinal homeostasis and B-cell development were still compromised in *Adar1p150*$^{-/-}$*/Mavs*$^{-/-}$ mice thereby attributing these specific roles to ADAR1p150. [43] In conclusion, these experiments suggested that the two ADAR1 isoforms have distinct roles with ADAR1p110 being integral for kidney patterning, while ADAR1p150 being the major isoform responsible for ADAR1-knockout lethality (due to aberrant immune response), as well as intestinal homeostasis and B-cell development.

Despite great advancements in the field and intensive research on ADAR1-knockout lethality several questions remain unanswered (or partially answered) to date:

1) What is the functional role of the ADAR1p110 isoform? An insightful recent study by Dr. Nishikura's group suggests that ADAR1p110, which normally resides in the nucleus, under stress conditions gets phosphorylated and exported in the cytoplasm, orchestrating apoptosis of stressed cells in a Staufen1-dependent manner. [46]

2) Which cells contribute most to the lethal phenotype? Conditional inactivation-based strategies of ADAR1 in different cell types may provide further insights in this direction.

3) Is ADAR1 essential for all cell types and, if so, is the ADAR1p150/MDA5/IFN axis conserved in every cell type? For example, there are findings pointing out the importance of ADAR1-RNA editing-independent function. [47]

4) Is MDA5 the primarily involved dsRNA sensor in every cell type or are other sensors involved depending on tissue or even disease context? Of note, *Adar1*$^{-/-}$/*RIG1*$^{-/-}$ double mutant embryos were not recovered. [43]

5) Which are the involved dsRNAs transmitting “danger” signals?

Further studies are warranted to address these questions in future.

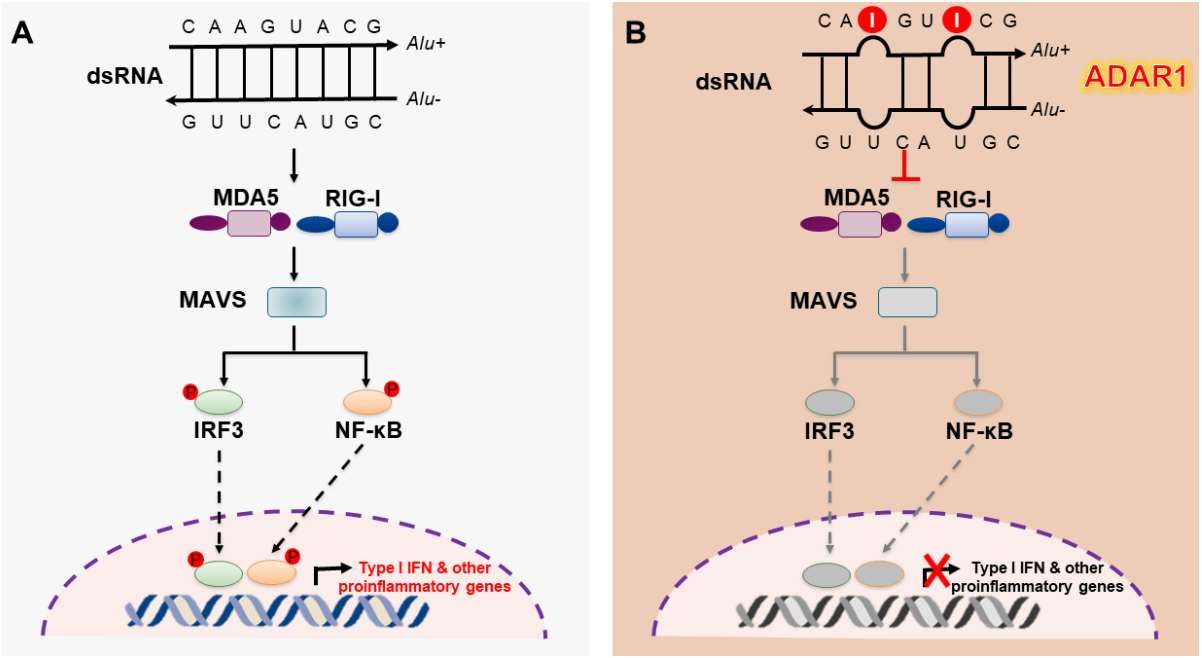

Figure 5. Simplified schematic representation of the role of ADAR1-induced RNA editing in the suppression of innate (auto)immune response. ***(A)*** *dsRNA is recognized by double strand RNA sensors (dsRNA sensors), namely MDA5 and RIG-I. Upon activation, both RIG-I and MDA5 receptors signal through MAVS adaptor, which in turn leads to the activation of NF-kB and IRF3/7 and subsequent upregulation of type I IFN and other proinflammatory genes thereby priming the innate immune response.* ***(B)*** *ADAR1 edits long dsRNAs located in the cytoplasm suppressing the downstream recognition by MDA5 or RIG-1. This event leads to overall suppression of innate immune response.*

Table 1. Summary of Adar-knockout mouse phenotypes				
Genetic model	**Day of mortality**	**Phenotype**	**Gene expression profiling**	**Ref.**
Adar$^{+/-}$ (genetic deletion of exons 12-13 of the deaminase domain)	E12.5-14.5	Severe defects in erythropoiesis with persistent presence of nucleated cells at E12.5		[29]
Adar$^{-/-}$ (genetic deletion of exons 12-15 of the deaminase domain)	E11-12	Pale appearance of the embryo possibly due to hemorrhage or defects in erythropoiesis and widespread apoptosis	Induction of interferon-stimulated genes (ISGs)	[28]
Adar$^{-/-}$ [genetic deletion of exons 7-9 (part of dsRNA binding domain + deaminase) or exons 2-13 (Z-DNA binding, dsRNA binding and part of deaminase domain)]	E11.5-12.5	Pale appearance of the embryo, liver disintegration and hematopoietic cell apoptosis	Induction of ISGs	[27]
Adarp150$^{-/-}$ (genetic deletion of exon 1A)	E11.5-12	Embryos show an abnormal morphology with disintegration of fetal lever and defects in erythropoiesis	Induction of ISGs	[43,44]
Adar1$^{E861A/E861A}$ (knock-in inactivating mutation in the catalytic domain)	E13.5	Embryos appear developmentally delayed with small liver and failure in erythropoiesis possibly due to the loss of erythroblasts	Induction of ISGs	[30]
Adar$^{-/-}$ / *Ifnar1*$^{-/-}$	E15.5	Fetal liver disintegration and defects in erythropoiesis		[37]
Adar$^{-/-}$ / *Mavs*$^{-/-}$ and *Adar*$^{-/-}$ / *Ifih1*$^{-/-}$	P1-P5	Alteration of kidney architecture, intestinal lesions, lack of organization in lymphoid follicles and defects in B cells development	ISGs expression is restored to wild type level	[37,43]
Adarp150$^{-/-}$ / *Mavs*$^{-/-}$	-	Double KO mice appear smaller than control littermates with alteration in intestinal homeostasis, lack of lymphoid follicle organization and defects in B-cell development	ISGs expression is restored to wild type level	[43]
Adar1$^{E861A/E861A}$ / *Ifih1*$^{-/-}$	-	Normal phenotype (slightly smaller size)	ISGs expression is restored to wild type level	[30]

Editing of the viral genome and functional consequences in human viral infections

Except for its role in “marking” endogenous RNA as “self” and preventing the inappropriate activation of the innate immune system, ADAR1 has a central role in the defense against viral infections. [48] Apart from editing of the genome of dsRNA viruses, ADAR1 can also affect any virus that forms dsRNA in some phase of their replication. ADAR1 can exert its effects on viral replication either by editing the viral genome or by directly binding to the viral dsRNA, as well as by interacting with other interferon-induced components of the innate immune system such as Protein Kinase RNA-activated (**PKR**). [49] Of interest, ADAR1-mediated A-to-I editing can have either proviral or antiviral effects, depending on the substrate virus as well as on the levels of ADAR1 expression. [50] **(Table 2)** In the following section, I will address and briefly summarize the best-studied editing-mediated effects of ADAR1 on the replication and infectivity of human viruses, as well as their implications for human disease.

The ability of ADAR1 to edit viral dsRNA was first described in Hepatitis Delta Virus (**HDV**). [51] The majority of editing events in the HDV-antigenome are detected at the “amber/W site” (adenosine 1012) localized in the mRNA of the HDV-antigen (HDAg), where a substitution of a stop codon by tryptophan leads to the formation of a larger isoform of the HDAg (HDAg-L). [52] HDAg-L promotes the packaging of the virus in particles and inhibits further viral RNA synthesis proposing a central role for RNA editing in viral life cycle. [53] However, when overexpressed, both ADAR1 and ADAR2 can inhibit viral replication and viability, underscoring the importance of tight regulation of RNA editing for HDV. [54] Normally, ADAR1p110 seems to be the main editor of the HDV-antigenome, indicating that HDV-editing mainly occurs in the host cell’s nucleus. [55] However, in the case of IFNα treatment, i.e. when dealing with co-existing Hepatitis B infection, ADAR1p150 becomes the main editor of HDV

antigenome. [56,57] In accordance with its antiviral role during IFNα treatment of HDV infection, ADAR1p150 has been shown to inhibit HCV replication. *In vitro* silencing of ADAR1 led to substantial increase in HCV replicons, while IFNα treatment of Huh-7 cells and subsequent upregulation of ADAR1p150-mediated A-to-I editing partially inhibited viral protein synthesis and replication. [58] Since pegylated IFNα confers a valuable therapeutic option in chronic hepatitis C, an editing-dependent ADAR1-mediated mechanism of viral control may be of relevance *in vivo*.

Another example of the pivotal role of ADAR1 in viral infection comes from studies on Human Immunodeficiency Virus (**HIV**), the majority of which propose a proviral role of ADAR1 in HIV protein synthesis, replication and infectivity. [59,60] Phuphuakrat *et al.* first described that ADAR1 overexpression in HEK293T and COS-7 cells upregulated HIV-p24 gag protein expression through an editing-dependent mechanism. Of interest, ADAR1 overexpression induced site-specific editing of the *env* gene, which encodes the protein of the viral envelope. [61] Further *in vitro* and *ex vivo* experiments using immortalized cell lines (HEK293T, Jurkat T cells) or primary cells (peripheral blood mononuclear cells, $CD4^+$ T cells) also proposed a role for ADAR1-mediated A-to-I editing in the release of HIV viral particles and viral infectivity. [59] ADAR1 may also render T-cells susceptible to HIV infection, since stimulated $CD4^+$ T cells [*ex vivo* stimulation of primary HC-derived $CD4^+$ T cells with phytohemagglutinin (PHA)], which are more prone to HIV infection compared to resting T-cells, overexpressed ADAR1. [61] On the other hand, Biswas et al. proposed that ADAR1-mediated editing has a negative effect on HIV protein synthesis and infectivity due to inefficient transportation of edited RNA from the nucleus to the cytoplasm and subsequent inhibition of viral protein synthesis. [62] Whether ADAR-mediated editing finally exerts a prominent pro-

or anti-viral effect on HIV or if editing-independent mechanisms such as PKR-inhibition are implicated in the control of HIV [63,64] remains to be elucidated in future studies.

Furthermore, Measles Virus (MV) offers an excellent example of conditional manipulation of RNA editing in favor of viral replication and infectivity. While ADAR1-mediated editing does not seem to affect acute MV infection [65], an important role for A-to-I RNA editing has long been suggested for measles viral infection complicated by subacute sclerosing panencephalitis (SSPE). In the first report, Baczko et al. isolated a defective M protein from the brain of four affected individuals that escaped recognition from the immune system, [66] which later on was found to include a large number of A-to-G hypermutations (suggestive of ADAR-mediated A-to-I editing). [67]

Finally, ADAR1-mediated editing has been implicated in the regulation of the viral replication of various other viruses including influenza A, vesicular stomatitis virus, lymphocytic choriomeningitis virus, Epstein-Barr virus, human herpesvirus 8 and respiratory syncytial virus. **(Table 2)** Overall, a wealth of preclinical evidence *(ex vivo, in vitro* and *in vivo*) suggest a central role for ADAR1-mediated RNA editing in human viral infection. ADAR-induced “mutations” in the viral antigenome alter the structural proteins of the viruses affecting their ability to replicate and infect host cells, as well as to escape immune recognition. MicroRNAs that may activate or suppress the innate immune system comprise also targets of ADAR-mediated editing. [68] Whether A-to-I editing could be exogenously manipulated to serve as a therapeutic target in chronic viral infections remains to be examined in future studies.

Table 2. The role of ADAR1 and ADAR2 in viral infections

Virus	Main "editor"	Model of study	Editing sites	Effect	Treatment - ADAR overexpression/ silencing	Key findings	Ref.
EBV	ADAR1	*In vitro* Lymphoblastoid-,Daudi Burkitt lymphoma- and nasopharyngeal Ca cells, HeLa, HEK293T cells	Pri-miRNAs	Antiviral		A-to-I editing inhibited wild-type miR-BART6-5p "loading" in the miRNA-induced silencing complex. Editing of a mutated pri-miR-BART6 in Burkitt/nasopharyngeal carcinoma cell lines inhibited its processing.	[68]
HCV	ADAR1 p150	*In vitro* Huh-7 cells	Radio-labeled AMP	Antiviral	IFNα treatment	Increased A-to-I editing of radiolabeled AMP. Inhibition of HCV replicon synthesis.	[58]
					siADAR1	5- to 41-fold increase of HCV replicons.	
					siADAR2	No effect on HCV replicons.	
HDV	ADAR1	*In vitro* Huh-7, HEK293 cells	HDAg (A1012) ("Amber/ W" site)			HDAg-L production Switch from replication to packaging	[51,52,69]
	ADAR1	*In vitro* Huh-7, HEK293 cells	HDAg (A1012) ("Amber/ W" site)		siADAR2	No effect on editing of HDV-antigenome.	[55,70]
					siADAR1p150	No effect on editing of HDV-antigenome during replication.	
					siADAR1	Inhibition of HDV-antigenome editing. Reduced production of HDV virions.	
	ADAR1/ ADAR2	*In vitro* Huh-7 cells	HDAg (A1012) ("Amber/ W" site)		IFNα treatment (ADAR1p150)	Increased editing of amber/W site. Increased HDAg-L production. Inhibition of HDV replication. ADAR1p150 is mainly responsible of IFNα-induced HDV RNA editing.	[54,56,57]
					ADAR1 overexpression		
					ADAR2 overexpression		
HHV-8	ADAR1	*In vitro* PEL, Rat-3 cells	K12 RNA	Anti-neo plastic		Increased editing of K12 RNA at 117990A during the switch from latent to lytic replication. Rat-3 cells transfected with edited K12 RNA showed decreased focus formation.	[71]
		In vivo nude mice				Nude mice injected with edited kaposin construct developed no tumors in contrast with 5/5 mice injected with the unedited construct	
HIV	ADAR1	*In vitro* COS-7, HEK293T cells	Env	Proviral	ADAR1 overexpression	Upregulation of p24 gag protein expression	[61]
					Catalytically-inactive ADAR1 overexpression	No effect on p24 gag protein expression	
					siADAR1	Downregulation of p24 gag protein expression.	
	ADAR1	*In vitro* HEK293T, Jurkat-T cells	5' UTR, Rev, Tat	Proviral -Viral infectivity	ADAR1 overexpression	Significantly increased release of HIV virions. Increased viral infectivity in primary human CD4+ T cells.	[59]

		Ex vivo Primary $CD4^+$ T cells			Catalytically-inactive ADAR1 overexpression	No effect on HIV virions' release or viral infectivity.	
	ADAR2	*In vitro* HEK293T, Jurkat T cells	5' UTR	Proviral	ADAR2	Significantly increased release of HIV virions. No effect on viral infectivity.	[72]
					Catalytically-inactive ADAR2	No effect on HIV virions' release or viral infectivity.	
					siADAR2	Impaired HIV protein synthesis.	
	ADAR1	*Ex vivo* PBMCs, primary $CD4^+$ T cells	na	Proviral		Decreased HIV protein synthesis and viral replication in AGS-compared to HC-derived PBMCs.	[60]
		In vitro Jurkat T cells			siADAR1	Inhibition of viral protein synthesis and replication.	
	ADAR1 p150	*In vitro* HEK293T, Jurkat T, HeLa cells *Ex vivo* Primary $CD4^+$ T cells	Rev, env	Antiviral	IFNα treatment (ADAR1p150)/ADAR1	Inhibition of HIV protein synthesis and viral infectivity.	[62]
					catalytically-inactive ADAR1 overexpression	No significant effect on viral protein synthesis or infectivity.	
	ADAR1 p150	*Ex vivo* Macro-phages	Envelope gp120 V3	Antiviral	IFN-γ treatment	Increased editing of the viral envelope RNA. Inhibition of HIV replication.	[73]
					siADAR1	Increased viral infectivity.	
					siADAR2	No effect on viral infectivity.	
Influenza A	ADAR1	*In vitro* HEK293T, A549 cells	Reporter plasmid	Proviral	ADAR1 overexpression	NS1-ADAR1 interaction increases ADAR1-mediated editing and viral protein expression.	[74]
					catalytically-inactive ADAR1 overexpression	Decreased viral protein expression.	
					siADAR1	Decreased viral protein expression and viral production.	
LCMV	ADAR1 p150	*In vitro* , *in vivo* L929 cells, C57BL6 mice	LCMV glyco-protein	Antiviral		Editing of LCMV glycoprotein led to frequent production of non-functional glycoprotein, partially reducing LCMV infectivity.	[75]
MV-SSPE	?	*In vivo*, YAC-CD46xRAG $^{-/-}$mice expressing MV-receptor	M protein of MV	Proviral		Intracerebral transfection of engineered-MV viruses with hypermutated M protein (resembling A-to-I edited protein) caused an SSPE-like disease in mice.	[76]
Measles Virus	ADAR1 p150	*In vitro* Vero, HeLa cells	Defective Interferin g (DI) RNAs	Proviral		Several MV DIs had a large number of A-to-G hypermutations, suggestive of ADAR1-mediated A-to-I editing.	[77]

1.4 A-to-I RNA editing in chronic inflammatory diseases

1.4.1 ADAR1-induced RNA editing in cancer

Editing of coding transcripts in solid tumors

In an elegant study, Chen *et al.* reported that the constitutively expressed nuclear ADAR1 isoform, ADAR1p110, edits antizyme inhibitor 1 (**AZIN1**) pre-mRNA shifting the cells towards a more tumorigenic phenotype, as studied in human hepatocellular carcinoma (HCC) specimens and in a tumor animal model. [78] In specific, the authors provide mechanistic insights supporting that a single recoding event (S367G) in the AZIN1 transcript induces a conformational change that leads to enhanced AZIN1-antizyme direct interaction and thereby increases the levels of two key regulators of the cell cycle, ornithine decarboxylase (ODC) and cyclin D1 (CCND1), augmenting in this way the tumor cell proliferation. [78] Compelling clinical evidence derived from 94 patients with HCC demonstrated increased RNA editing levels of AZIN1 mRNA in correlation with increased ADAR1 expression levels. [78] Another oncogenic target, filamin B (**FLNB**), is edited by both ADAR1 and ADAR2, promoting uncontrolled cancer cell proliferation *in vitro*. [79] Future studies may elucidate the functional role of filamin B in tumor pathogenesis *in vivo*.

Glioma associated oncogene 1 (**GLI1**) also holds a key role in cell proliferation during tumorigenesis and in embryonic patterning through Hedgehog signaling. [80,81] Of note, the nucleotide 2179 constitutes a highly edited site in *GLI1* mRNA that results in an amino acid substitution (R→G) and changes the GLI-transcription efficiency, thereby reducing GLI-dependent cellular proliferation. [81] Additionally, it has been reported that ADAR1-mediated RNA editing regulates GLI1-dependent Dyrk1a-mediated phosphorylation, resulting in decreased transcription and reduced medulloblastoma cell growth. [81] Another study has shown that ADAR1p110-induced

RNA editing of *Gabra3* results in the suppression of breast cancer migration, invasion, and metastasis due to the lack of active AKT-signaling. [82] Moreover, bladder cancer-associated protein (BLCAP), which is highly conserved among species, is subjected to RNA editing by both ADAR1 and ADAR2 in the highly conserved amino terminus, leading to amino acid changes and thus resulting in alternative protein isoforms. [83] BLCAP RNA editing levels have been reported to be decreased in astrocytomas, colorectal and bladder cancer. [83]

Editing of non-coding RNAs (miRNAs, lncRNAs)

It has been previously proposed that loss of ADAR1 promotes tumor growth in metastatic melanoma. [84] A few years later, Shoshan *et al.* documented that ADAR1 expression and function are impaired in metastatic melanoma by direct binding of the transcriptional factor CREB. [85] More importantly, the authors showed that ADAR1 edits miR-455-5p with functional consequences in an experimental model of melanoma growth and metastasis in mice. Specifically, the study suggested two distinct underlying mechanisms suggesting that ADAR1-dependent RNA editing of pri-miR-455 may result in: a) differential binding of Drosha thereby inhibiting its maturation process, and/or in b) altered miRNA targetome (re-targeting) since edited miR-455-5p recognizes a different set of transcripts than the unedited miR-455-5p, which targets the tumor suppressor CPEB1 and thereby promotes melanoma metastasis. [85] Thus, ADAR1-RNA editing promotes CPEB1 expression by inhibiting miR-455-5p targeting of the CPEB1 transcript, resulting in the suppression of melanoma growth and metastasis. [85] Further studies are warranted to provide evidence whether this mechanism is conserved in humans and may be of clinical prognostic value. Given the central role of IFN in the treatment of melanoma, [86] which in turn is expected to lead

to increased ADAR1 expression, future studies are warranted to examine the prognostic role of the IFN/ ADAR1/ CPEB1 axis in metastatic melanoma.

Salameh and colleagues showed that the intronic long non-coding RNA Prostate Cancer Antigen 3 (lncPCA3), a promising biomarker for prostate cancer, binds to the pre-mRNA of Prune homolog 2 (PRUNE2) forming in this way a double-stranded RNA structure, which is edited by ADAR1. [87] A-to-I RNA editing of the *PCA3:PRUNE2* duplex results in the decreased expression of both RNAs, *lncPCA3* and *PRUNE2* mRNA. [87] In accordance with the prior documented PRUNE2 function as a tumor suppressor, the authors reported that this ADAR1-induced RNA editing-dependent mechanism augments malignant cell growth of prostate cancer cells. [87]

Another long-non coding RNA, *NEAT1*, which is essential for the paraspeckle nuclear body formation, [88,89] has been documented to hold a key role in tumorigenesis and chemosensitivity mainly by directly interacting with p53**.** [90–92] Interestingly, it has been previously reported that *NEAT1* directly interacts with $p54^{nrb}$ and actively participates in an ADAR1-RNA editing-dependent nuclear retention of the edited form of *Lin28* mRNA, which is preferentially fished out by $p54^{nrb}$, [93] confirming the initial observations of another group. [94] *Xist* (encoding X-inactive specific transcript) and *Malat1* (encoding metastasis-associated lung adenocarcinoma transcript 1) are two additional lncRNAs with a well-established link with tumorigenesis. [95,96] Nevertheless, whether direct RNA editing of these lncRNAs alters their molecular repertoire with functional consequences in tumor biology remains elusive.

Collectively, these findings underline the necessity of a balanced ADAR1-induced RNA editing during cancer progression. More importantly, these studies implied that ADAR1 targeting may offer a valuable option in various types of cancer.

Indeed, recent preclinical studies showed promising results from ADAR1-deletion in various cancer cells *in vitro* or *in vivo*. On one hand, cancer cells characterized by chronic tumor-derived IFN pathway activation were shown to be “primed” to respond to dsRNA accumulation, rendering them susceptible to ADAR1-deletion [97], while on the other hand ADAR1-knockout was shown to sensitize tumors to immune checkpoint blockade, probably through activation of MDA5 and PKR. [98] Future studies are warranted to transfer these findings from bench to bedside taking advantage of the multifaceted role of ADAR1 that underlies its great potential as a therapeutic target, while applying a targeted approach to avoid the detrimental effects of a global ADAR1 deletion.

1.4.2 ADAR1-induced RNA editing in cardiovascular disease

In 2020, cardiovascular disease (**CVD**) remains the primary cause of mortality in the world with the Global Burden of Disease study reporting that 1 out of 3 deaths worldwide are attributed to CVD, which is double than cancer-related mortality. [99] However, only recently RNA editing has been clearly linked to the pathogenesis of this important disease. [100]

Endothelial cells

In specific, our research group has recently described a previously unrecognized mechanism regarding ADAR1-induced clustered RNA editing within the 3’UTR of cathepsin S (**CTSS**) mRNA, a proinflammatory gene with a well-established role among others in vascular function [101] and atherosclerosis, [102] the main cause of CVD. The observed clusters of RNA editing lie within the 3’UTR of CTSS and

specifically within two inverted *Alu* repeats [100] which form double stranded RNA (dsRNA) regions, a prerequisite for A-to-I RNA editing. [8,103,104] RNA editing of the *Alu* dsRNAs within the *CTSS* 3'UTR induces bulges enabling Human Antigen R (**HuR**), a known single-strand RNA-binding protein, to bind and confer stability on the bound mRNAs. In this way, CTSS expression is increased at both the mRNA and protein level with functional consequences for the vascular endothelial cells. Of note, hypoxia and inflammation (TNFα, IFN-γ) were shown to increase ADAR1-induced RNA editing of CTSS. Due to primate-specificity of *Alu* elements precluding the use of an animal model, our group investigated the clinical implications of this mechanism directly in human tissues from >300 human subjects, reflecting various stages of CVD. We revealed that ADAR1-induced RNA editing of *CTSS* is a novel regulatory mechanism driving CTSS expression in all stages of CVD, including subclinical atherosclerosis, coronary artery disease, aortic thoracic aneurysms and advanced carotid atherosclerotic disease. Based on additional observations that a) HuR-binding motifs are enriched within the 3'UTRs of edited transcripts in close proximity to RNA editing sites and b) the expression of those transcripts is regulated by ADAR1, this mechanism may be of relevance for the expression of multiple targets, which remain to be identified and studied with regards to their functional role in cardiovascular or other chronic inflammatory diseases.

Vascular smooth muscle cells

A few more studies have reported interesting findings on the potential role of ADAR1 and A-to-I RNA editing in the pathogenesis of CVD. Smooth muscle cells show plasticity and in response to proatherogenic stimuli switch from a contractile to a proliferative phenotype promoting atherosclerotic plaque formation. On the other

hand, their presence within advanced atherosclerotic plaques may prevent the rupture of the fibrous cap and its tremendous consequences (reviewed in [105]). Interestingly, ADAR1-induced RNA editing may partially regulate phenotypic switch of smooth muscle cells, [106] but not the contractile SMCs. Specifically, the authors suggested that during platelet-derived growth factor (PDGF)-BB-induced phenotypic switch, pre-mRNAs of smooth muscle cell markers including myosin heavy chain (MYH11) and smooth muscle α-actin (ACTA2), undergo intronic RNA editing and are accumulated. Simultaneously, they observed that the mature mRNA and protein levels of these markers are decreased, potentially due to alternative splicing, leading thus to maintenance of SMC phenotypic switch, [106] given the downregulation of these genes is a hallmark of this process (reviewed in [107]). However, additional *in vitro* and *in vivo* studies clarifying the underlying mechanism (e.g. the presence of alternative spliced variants) are warranted to consolidate these interesting findings and define the role of ADAR1-induced RNA editing in smooth muscle cell biology, which could be causatively involved in CVD.

RNA editing-controlled changes in miRNA binding

RhoA and cell division control protein 42 homolog (Cdc42) are both members of the ubiquitously expressed Rho family. [108] Rho GTPase activating protein 26 (ARHGAP26) regulates the activity of RhoA by converting the active GTP-bound RhoA to the inactive GDP bound form. [109] Thus, it contributes to various Rho-related pathologies, such as CVD. [110] Of note, Wang *et al.* demonstrated that the 3' UTR of *ARHGAP26* is extensively edited by ADAR1 disrupting the miR-30b-3p and miR-573 binding sites onto the transcript and thus upregulating its expression and boosting the negative regulatory effect of ARHGAP26 on RhoA, as well as on its other target Cdc42,

a regulator of the cell cycle. [109,111] As a result, the absence of ADAR1-induced A-to-I RNA editing results in higher RhoA-GTP activity. [109]

Following a similar mechanistic approach, Nakano *et al.* reported that ADAR1 edits the 3'UTR of human aryl hydrocarbon receptor (AhR) mRNA [112]. The edited form of AhR harbors a recognition motif for miR-378 leading to increased miR-378-binding to *AhR* mRNA and ultimately in downregulation of AhR mRNA and protein levels. [112] Strikingly, AhR is causatively involved in atherosclerosis, since its activation has been shown to induce vascular inflammation and to promote atherosclerosis in *Apoe*$^{-/-}$ mice. [113] The findings of these studies lead us to hypothesize that ADAR1-AhR and/or ADAR1-ARHGAP26 axis could represent additional mechanisms through which ADAR1-induced RNA editing may be involved in the pathogenesis of atherosclerosis.

Taken together, these studies underline the multifaceted role of RNA editing as an additional regulatory layer in CVD. Identification of specific targets (e.g. cathepsin S, ARHGAP26, AhR) and editing sites along with recent technological advances (e.g. site-directed CRISPR/Cas9 genome editing or CRISPR/Cas13 transcript editing) may provide unique and targeted therapeutic opportunities in the era of precision medicine.

1.4.3 ADAR1-induced RNA editing affects DNA repair: implications for aging-related pathologies

Reactive oxygen species (**ROS**) comprise a major source of excessive oxidative stress, which is reflected by DNA damage accumulation and eventually cellular dysfunction (senescence) [114]. Among the consequences of ROS production is increased vascular tone, vascular permeability and in general, vascular endothelial

dysfunction, which constitute therapeutic targets for CVD (reviewed in [115]), cancer [116,117], systemic autoimmune diseases [118], and aging-related disorders. [119] Nei-like enzymes NEIL1-3 serve as DNA repair enzymes by excising oxidized base lesions (base excision repair mechanism of DNA), which are generated in excessive amounts in response to endogenous metabolic activities and oxidative stress. [120] Interestingly, ADAR1 was found to functionally regulate DNA repair by editing *NEIL1* pre-mRNA. [121,122] In specific, ADAR1-induced RNA editing causes an amino acid switch (K242R) harbored into the lesion recognition loop of the enzyme. The authors suggested that this recoding event renders the edited version of NEIL1 more efficient in removing a particular oxidized base lesion, namely guanidinohydantoin (Gh), while the unedited form preferentially removes thymine glycol (Tg) oxidized base lesions [122]. Nevertheless, it is still unclear how exactly RNA editing facilitates the altered properties of the enzyme and therefore further molecular and structural studies would deepen the understanding of this important mechanism.

Given that both forms of the enzyme, bearing distinct properties, are present in the cells, [122] RNA editing can be considered as an endogenous on-off switch on NEIL1 which can be rapidly accessed in response to cell stimuli and thereby modulate DNA repair. Interestingly, Yeo *et al* reported that *NEIL1* RNA editing is significantly induced after IFN-α treatment of human glioblastoma cells due to the upregulation of the IFN-inducible isoform ADAR1p150. [16,122] Along with a recent observation that ADAR1 levels are upregulated by H_2O_2 in neonatal cardiac myocytes, [123] it would be tempting to hypothesize an imperative role for ADAR1 in oxidative stress cascade and aging-related disorders.

Furthermore, in an elegant report, Dr. Nishikura's group showed that silencing of ADAR1 downregulated two central molecules in DNA double strand break repair

(DSB/R)- the most significant DNA repair mechanism for cell viability, namely ATM and RAD51, promoting apoptosis [46]. Similarly, in a recent report ADAR1 was shown to control the stability of multiple DDR components and knock-down of ADAR1 led to increased apoptosis of breast cancer cells *in vitro*. [124] The interplay between RNA editing and DNA damage response in the context of chronic inflammatory and autoimmune diseases, given the bi-directional relationship of both mechanisms with the innate immune response, warrants further investigation.

1.5 A-to-I RNA editing and systemic autoimmune diseases: what is known to date?

Systemic autoimmune diseases are classically characterized by auto-antibody production (adaptive immune response) against self-antigens (autoantigens) leading to tissue damage. However, in the past decades innate immune response has gained significant attention as part of systemic autoimmune disease pathogenesis. [125] Recognition of nucleic acids by innate immune receptors (TLRs and non-TLRs) leading to innate immune response (type I IFN and NF-kB pathway activation) has been shown to be implicated in the initiation and perpetuation of the systemic autoimmune diseases. [125,126] Specifically, type I IFN pathway activation has been implicated in the pathogenesis of various autoimmune diseases including Systemic Lupus Erythematosus (**SLE**), the prototypical autoimmune disease [127], Systemic Sclerosis[128] and Sjogren's Syndrome, [129] while recently it has been recognized as a key pathophysiological aspect of early RA [130]. Of note, a monoclonal antibody directed against the common type I IFN receptor (IFNAR) recently showed clinical efficacy in a phase III trial in patients with active SLE. [131]

The pivotal role of ADAR1 in prevention of an aberrant innate immune response (as shown in the multiple mouse models presented in earlier chapters) together with its induction by inflammatory cytokines such as TNFα or IFN-γ, [100,132] which are augmented during systemic autoimmune and other inflammatory diseases, designate ADAR1-induced RNA editing as a worth-exploring factor in the pathogenesis of autoimmune diseases.

ADAR1 and type I interferonopathies

The potential role of ADAR1 in the pathogenesis of systemic autoimmune diseases was first derived from Aicardi-Goutières syndrome (**AGS**), a rare genetic type I interferonopathy, usually manifested as an early-onset encephalopathy, occurring due to different genetic causes all of which lead to aberrant type I IFN expression. [133–135] In accordance with the role of ADAR1 as a suppressor of type I IFN-signaling, Yanick Crow's group first reported a census of mutations in *Adar1* genetic locus in patients with AGS, the majority of which resulted in amino-acid substitutions within the catalytic (deaminase) domain of the enzyme. [136] A few years later, the same group showed that gain-of-function mutations in *IFIH1 (MDA5)* gene locus result in an induction of type I IFN signaling, [137] in line with the suggested role of ADAR1/MDA5/IFN axis in innate immune response. AGS-causing mutations were mapped to the ADAR1 deaminase domain enabling the modelling of how exactly these mutations interfere with the interaction of ADAR1 with the RNA substrates [138] consolidating further the functional consequences of these mutations in AGS.

Bilateral striatal necrosis (**BSN**), another pediatric "interferonopathy" which may co-exist with AGS, [139] is clinically characterized by dystonic movement and has also been recently associated with *Adar1* mutations. [140] Strikingly, BSN patients with an *Adar1* mutation exhibit a prominent type I IFN signature in contrast with those patients who had no mutation present in *Adar1* locus. [140] Interestingly, a case of unexplained spastic paraplegia was also attributed to the presence of *Adar1* mutation. [141]

Table 3. Mutations in ADAR1 and associated disorders (selected publications)				
Disease	**Main editor**	**Population (patients)**	**Effect**	**Reference**
Type I Interferonopathies				
Aicardi-Goutières Syndrome (AGS)	ADAR1	12	7/8 mutations located in ADAR1 deaminase domain. Increased IFN-induced gene expression probably due to defective ADAR1-mediated RNA editing.	[136]
Bilateral Striatal Necrosis (BSN)	ADAR1	13	Increased IFN-induced gene expression in 7/7 assayed BSN patients with ADAR1 mutations compared with 0/4 BSN patients with normal ADAR1 sequence.	[140]
AGS+BSN	ADAR1	2	Co-existence of BSN and AGS in patients with ADAR1 mutations.	[139]
Spastic paraplegia	ADAR1	5	Mutations in ADAR1 (1), IFIH1(1), RNASEH2B(3). Same ADAR1 mutation had previously been reported in patients with AGS, BSN or DSH. ADAR1/IFIH1 mutations were associated with significantly elevated type I IFN-induced gene expression, while RNASEH2B not.	[141]
Dyschromatosis symmetrica heredtaria (DSH)	ADAR1	55	7 ADAR1 mutations detected among familial and sporadic DSH cases.	[142]

Implications in type I IFN-mediated systemic autoimmune diseases

Elevated levels of ADAR1 and particularly of ADAR1p150 isoform have been previously reported in peripheral blood mononuclear cells (PBMCs), T lymphocytes and Natural Killer (NK) cells isolated from patients with SLE. [143–146] Laxminarayana et al. described increased ADAR1 expression in lupus T lymphocytes, [144] as well as altered RNA editing of ADAR2 mRNA [147] more than 10 years ago, however until recently no further studies on the involvement of RNA editing in systemic autoimmune diseases were published.

Alu editing

In 2018, Roth et al. performed a transcriptome-wide analysis of RNA editing events located in Alu elements (Alu editing index-AEI) in patients with SLE. In their study the researchers showed increased expression of ADAR1 in whole blood samples (PAXgene tubes) derived from SLE patients compared to controls and specifically in those patients where an increased type I IFN signature was present. Further, increased Alu RNA editing rate (AEI) was detected in patients with a high type I interferon signature, but also in those with low (comparable to controls) type I IFN induced gene expression, although to a lower extent. [148] Surprisingly, the same research group found a decrease in Alu RNA editing rate in keratinocytes derived from psoriasis patients compared to controls, [149] despite significantly increased ADAR1 expression in association with increased expression of proinflammatory cytokines in the same cells.

These discrepancies between SLE whole blood samples and psoriatic keratinocytes, both characterized by an inflammatory environment, may be attributed to various factors, including:

i) the tissue- and cell-specificity of A-to-I RNA editing [150]

ii) the tissue/cell-specific presence of additional factors that may interfere with the ability of ADARs to edit dsRNA [151]

iii) the possibility that the Alu editing index may reflect not only ADAR1, but also ADAR2 editing activity, as described by Eisenberg, Levanon and colleagues recently. [152]

Indeed, another research group recently showed that the RNA binding protein Ro60 can directly interfere with ADAR1's editing activity and thus hypothesized that the presence of auto-antibodies against Ro60 (often seen in patients with SLE or primary

Sjogren's syndrome) may be partially responsible for the increased RNA editing levels observed in the blood of SLE patients. [151]

A-to-I RNA editing as a source of autoantibodies

As already described earlier the vast majority of A-to-I RNA editing takes place in non-coding regions and specifically in Alu elements, while only a very small proportion are located in coding sequences. However, the presence or absence of a single RNA editing event in a coding region can have detrimental effects as suggested by the 100% lethality of ADAR2 (*Adarb1*)-knockout mice, which is rescued by reconstitution of the edited form of the glutamate receptor. [153] Of interest, in their study Roth and colleagues showed that increased RNA editing is also observed in the transcribed region of multiple mRNA molecules (recoding events) in SLE patient samples, potentially leading to the production of novel proteins falsely recognized by the immune system as auto-antigens and thus ultimately to auto-antibody production . [148] The same notion has been shown in cancer, where RNA editing has been suggested to produce neo-epitopes leading to activation of $CD8^+$ T cells specific for the edited peptides. [154]

In conclusion, RNA editing being itself induced by inflammatory mediators may serve as "oil on fire" leading to stabilization of proinflammatory molecules as well as generation of neo-antigens and auto-antibody production, or in other cases as a "break" of the immune response. This complex, multifaceted contribution of RNA editing in the chronic, auto-inflammatory response led us to conduct the current study.

2. Rheumatoid Arthritis

Introduction

Rheumatoid Arthritis (**RA**) is a chronic inflammatory disorder which affects approximately 1% of the population. [1] It primarily affects small and medium-sized joints in a symmetrical pattern, but also has various extra-articular features including lung involvement, rheumatoid nodules, vasculitis, as well as increased co-morbidities, such as cardiovascular disease due to accelerated atherosclerosis. [1,155] Despite significant therapeutic advances with the introduction of biological treatments and new therapeutic approaches (treat-to-target) in the past decades, remission rates in RA remain unsatisfactorily low even under treatment with biologic disease-modifying anti-rheumatic drugs (**bDMARDs**). [2] Moreover, even when patients achieve drug-free remission, approximately half relapse within the next 6 months. [3] Therefore, there is an emerging need for identification of the underlying disease mechanisms that may help both the development of novel prognostic biomarkers that will predict response to current therapies and the discovery of novel therapeutic targets.

2.1 Epidemiology and risk factors

RA affects approximately 0.5-1% of the European population, showing a gradual increase in prevalence from southern European countries (3.3 cases per 1000 population) to northern European countries (5.0 cases per 1000 population). [156] RA primarily affects women (3:1 women/men ratio) and peak incidence is observed in men aged over 70 years and in women between 50 and 60 years of age. [157] In a recent analysis of the Greek country-wide prescription electronic database covering 10.223.000 Greek citizens by Sfikakis et al., among the 9,824 RA patients identified to be currently under biological treatment, the mean age was 61 years and a 4:1 women to men ratio was observed. [158]

RA is a complex disease with both genetic and environmental risk factors contributing to disease development and progression. Twin studies support a strong genetic component estimating the heritability of RA (phenotypic variation attributable to genetic variation in a certain population) at approximately 60% [159], especially for anti-CCP positive patients. The strongest genetic risk factor is HLA-DRB1 (especially HLA-DRB1*01 and HLA-DRB1*04), which may account for approximately 30% of the total genetic risk of RA. [160] Many more genetic risk factors including PTPN22, [161] IL6ST[162] and IRF5 [162] polymorphisms have been identified. Some of these genetic predisposing factors are shared among seropositive and seronegative patients, while others are specific for only a subgroup. [155] More importantly, many of the genetic predisposing factors also predict a more severe disease course [155], or may affect response to currently available treatments. [160] Given the wide availability and decreasing cost of NGS (next generation sequencing) a better understanding of these polymorphisms and their effect on disease course or therapy

response could translate them into useful tools for risk stratification of patients with early arthritis.

On the other hand, environmental risk factors seem to also be integral in RA development with smoking being the most important contributing risk factor. Smoking is not only associated with increased risk of disease development (>2 times higher risk for ≥20 pack-year smoking history among men, reaching up to 4-fold increased risk for seropositive disease in current smokers [163]), but is also an adverse prognostic factor among RA patients being associated with worse clinical response to current therapeutic modalities, such as anti-TNF. [164] Multiple more environmental risk factors, such as periodontal disease, have been associated with increased disease risk potentially through development of citrullinated peptides that may ultimately lead to the production of tissue-damaging anti-CCP antibodies. [165,166]

Recently, epigenetic modifications such as DNA methylation, histone (de)acetylation and local chromatin (de)condensation have gained significant attention as part of the pathogenesis of RA and other systemic autoimmune diseases, acting in parallel or interacting with genetic and environmental risk factors (a nice review on RA epigenetics can be found in [167,168]).

2.2 RA pathogenesis

RA is a chronic disorder characterised by both systemic and localised (synovial) inflammatory response. [155] The primary lesion in RA is synovitis whereby infiltrating immune cells and hyperplastic synovial cells form a tissue called “pannus” and invade the normally nearly acellular synovium leading to cartilage destruction and later on to bone erosions. [169]

Autoantibodies

The presence of autoantibodies against an autoantigen is the cardinal feature of autoimmune disease. The first autoantibody detected among RA patients was the rheumatoid factor (**RF**), which is mostly present in the IgM form, and targets the Fc part of IgG antibodies. However, RF is quite common among patients with other systemic autoimmune diseases and in approximately 10% of the “healthy” elderly population, therefore limiting its value in disease diagnosis. [170]

Anti-cyclic citrullinated peptide (**anti-CCP**) auto-antibodies are also frequently found among patients with RA (60-70%) and have been associated with adverse disease prognosis. [171] Anti-CCP antibodies have a high specificity for RA (>95%) and can therefore help in differential diagnosis.

Systemic inflammation- Leukocytes

RA is characterised by both aberrant innate and adaptive immune responses. The role of T lymphocytes, the main components of the cell-mediated adaptive immune response, has been extensively studied in RA. A first hint that $CD4^+$ T-cells may be central in RA pathogenesis is derived from the strong association of HLA-DRB1 with disease development.

Currently, an antibody against CTLA4 (cytotoxic T-lymphocyte-associated protein 4) is among the biological therapies available for RA. B lymphocytes, mediating humoral adaptive immune response, are also integral components in RA pathogenesis being responsible for the production of auto-antibodies. [172] Anti-B-cell therapies (anti-CD20) are also currently among the therapeutic options in RA. [173]

Moreover, innate immune cells have also gained significant attention in the pathogenesis of RA. Neutrophils are central in the pathogenesis of RA through the release of cytotoxic and immunoregulatory molecules and have been shown to be critical in mouse models of RA, for initiation and progression of disease. [174] Moreover, through the excretion of extracellular traps (NETs) they may contribute to anti-CCP formation, [174] while a role as major source of type I IFN in early RA has also been suggested. [175]

Synovial inflammation

Synovium is the “target tissue” in RA. Normally the synovial membrane consists of a 1-3 cell-thick layer that produces nutrients and lubrication indispensable for the normal function of the joints. [176–178] However, in RA the synovium becomes hyperplastic, fibroblast-like synoviocytes proliferate and cluster in an invading tissue called “pannus”, [169] while white blood cells also infiltrate the synovium produce inflammatory mediators (TNF, IL-1, IL-6) and matrix degrading enzymes (matrix metalloproteinases, cathepsins) further aggravating synovial inflammation. Chronic synovial inflammation leads to cartilage degradation and, later on, to bone erosions.

Recently, researchers identified 3 major histological types of synovitis:

i) lympho-myeloid (B cells in addition to myeloid cells);

ii) diffuse-myeloid (myeloid lineage predominance, poor in B cells) and

iii) pauci-immune characterised by very few immune cells and prevalence of stromal cells.

Of note, the pauci-immune type was linked to lower disease activity and slower radiographic progression after 6 months in a recent large study. [179]

Moreover, with the recent development of single-cell RNA sequencing researchers were able to delineate the cellular landscape of the arthritic synovium and attribute the source of central inflammatory mediators, like IL-6, to specific cell sub-populations. [180] Moreover, the researchers identified multiple subsets of synovial fibroblasts with contradicting roles in synovial inflammatory response. [181] Whether therapeutic targeting of specific cell-subpopulations that mediate joint destruction will be achieved with modern technologies like liposomal nanocarriers remains to be elucidated in the near future.

Of interest, a recent pioneering study showed that proarthritogenic fibroblasts enter the circulation and “metastasize” arthritis to distant joints [182]. These fibroblasts increased in number before disease flares and decreased during the flare suggesting that they were recruited to the joint [182]. These findings validate previous work in mouse arthritis models [183–185] and open a new era, where mesenchymal cells may be considered the “drivers” of RA pathogenesis not only locally at the joints but also in the systemic circulation.

Figure 6

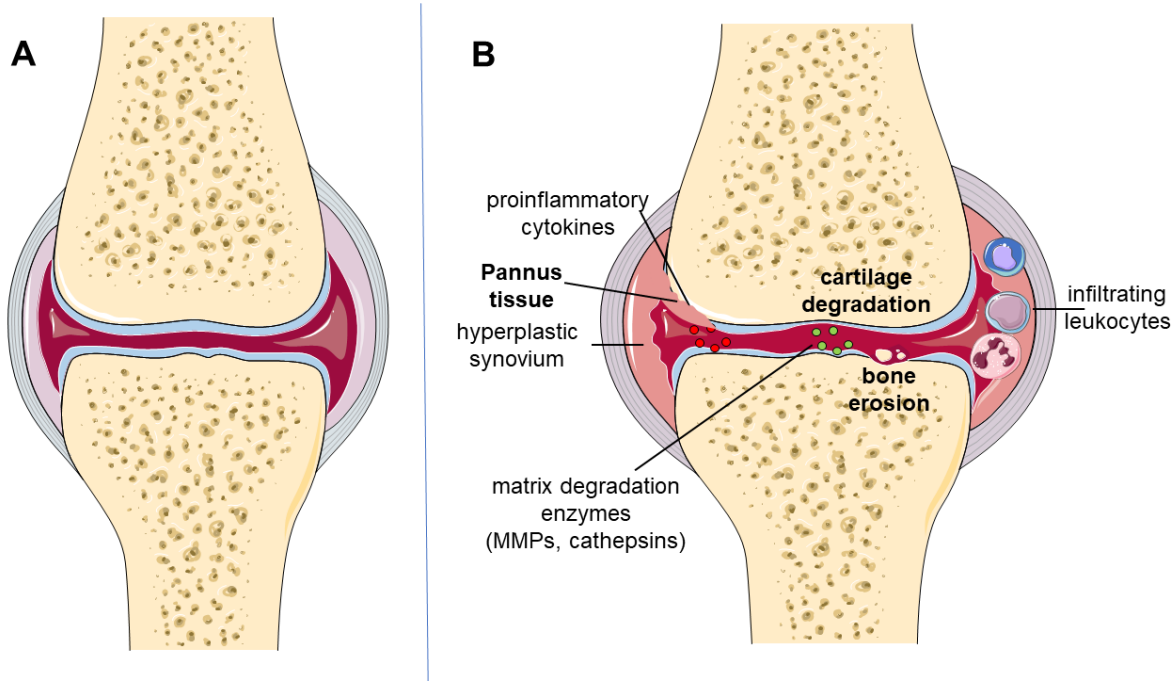

Figure 6. Normal and inflamed joint. ***A.*** *Normally the synovial membrane consists of a 1-3 cell-thick layer that produces nutrients and lubrication indispensable for the normal function of the joints.* ***B.*** *In RA the synovium becomes hyperplastic, fibroblast-like synoviocytes proliferate and cluster in an invading tissue called "pannus", while white blood cells also infiltrate the synovium and start producing inflammatory mediators (TNF, IL-1, IL-6) and matrix degrading enzymes (matrix metalloproteinases, cathepsins) further aggravating synovial inflammation. Chronic synovial inflammation leads to cartilage degradation and, later on, to bone erosions. Certain items on this figure have been adapted from Servier Medical Art by Servier (https://smart.servier.com – licensed under Creative Commons Attribution 3.0 Unported License)*

2.3 Proinflammatory gene expression in RA

Rheumatoid arthritis is characterized by chronic activation of immune cells and production of proinflammatory cytokines. Until recently, most studies on the regulation of proinflammatory gene expression in RA focused on the role of TNF and NF-kB-mediated transcriptional regulation of proinflammatory genes. Recently, an increasing number of studies suggest that type I interferons, the “antiviral” component of innate immune response classically associated with SLE, Sjogren’s syndrome and Systemic Sclerosis [128,129], may also be an integral part of RA pathogenesis.

Type I IFN-induced gene expression, the so called “interferon signature”, is detected in the blood of approximately 30-40% of patients with early RA [130,175] and polymorphonuclear cells seem to be the main contributors. [175] A high type I IFN-induced gene expression among patients with early arthritis has also been suggested to predict progression to RA. [186] Moreover, low type I IFN score pre-treatment has been associated with better response to B-cell depletion therapy in patients with RA, [187] while IFNβ/α ratio has had contradicting value for the prediction of anti-TNF response. [188,189]

Moreover, epigenetic alterations have recently gained significant attention as regulators of gene expression in various cell types in RA. A recent genome-wide methylation analysis in peripheral blood mononuclear cells (**PBMCs**) of RA patients revealed 1,046 DNA methylation positions associated with RA. [190] Of interest, when DNA methylation data were associated with gene expression, an interferon-inducible gene interaction network involving MX1, IFI44L (which are classically measured as part of the type I interferon signature) was associated with RA, further supporting the potential involvement of type I IFNs in RA pathogenesis. [190] Another recent study

showed that site-specific DNA methylation may regulate gene expression in T and B-cells from RA patients, including the IL6ST subunit of IL6-receptor,[191] a pathway with multiple clinical implications in RA and other systemic autoimmune diseases.

Histone (de)acetylation and its effect on chromatin organization has also been extensively studied in RA. E*x vivo* anti-TNF treatment of T-cells from RA patients has been shown to alter histone acetylation, thus, potentially affecting chromatin organization. [192] Accordingly, treatment of fibroblast-like synoviocytes or PBMCs derived from RA patients by histone deacetylase inhibitors *ex vivo* led to significant downregulation of IL-6 production, [193–195] while such drugs ameliorate collagen-induced arthritis. [196] More importantly, Givinostat, an oral histone deacetylase inhibitor was beneficial in children with systemic-onset juvenile idiopathic arthritis. [197]

2.4 Unmet clinical needs in treatment of patients with RA

Multiple therapies have been developed to date to tackle the plurality of immune responses and of the different immune cells mediating tissue damage in RA. Anti-cytokine treatments (anti-TNF, anti-IL6(R), anti-IL1) and cell-specific treatments (anti-CD20 for B lymphocytes, anti-CTLA4 for T lymphocytes) have revolutionized the course of this debilitating disease. [1] More recently, JAK-inhibitors have gained significant ground in the treatment of RA offering an additional choice equal to bDMARDS for patients not achieving satisfactory disease control with csDMARDS. [198,199]

Recently researchers have also started to develop synovial-targeted therapies either by directly targeting FLS [200] or by “carrying” immunosuppressive drugs with specialised carriers such as liposomal nanoparticles coated with tissue-specific antigens for local distribution of immunosuppressive drugs in the joints to avoid systemic immunosuppression. [201]

The development of new drugs, a uniform treat-to-target approach and the introduction of more sensitive criteria [202] enabling the early detection and treatment of RA have significantly improved the outcome of this disabling disease. [155] However, despite intensive research efforts, disease remission is only achieved in approximately half of patients, with a recent study reporting that only 1 out of 4 patients with RA achieved sustained (>1 year) disease-free remission in a large cohort of early arthritis. [2]

Two possible explanations for the limited efficiency of current therapies are that: 1) current therapeutic modalities have been developed in mouse models of arthritis thus disregarding any human-specific regulation of inflammation and

2) current biological therapies target specific cytokines (i.e. TNFα, IL-6, IL-1) rather than upstream mediators/regulators of the inflammatory response.

To tackle both aforementioned points herein we set to explore the role of Alu RNA editing in RA, a fundamental, primate-specific mechanism controlling proinflammatory gene expression at the post-transcriptional level. [14,19] RNA editing takes place mainly in non-coding regions of RNA and is a widespread phenomenon, particularly in the human transcriptome, largely due to the presence of inverted Alu repeats which altogether account for approximately 10% of the human genome. [21] The widespread Alu elements have recently emerged as critical regulators of inflammation and an enrichment of Alu elements has been recently reported in autoimmune diseases. [203,204] However the role of Alu RNA editing in human disease remains to date largely unknown. The current study aimed to explore the role of Alu RNA editing in regulation of proinflammatory gene expression in RA.

EXPERIMENTAL AND CLINICAL DATA

(ΕΙΔΙΚΟ ΜΕΡΟΣ)

Objectives

The current doctoral thesis aimed to address the following points:

1) Is A-to-I RNA editing deregulated in a prototypic chronic high-grade inflammatory disease such as RA?

2) Is A-to-I RNA editing involved in the regulation of expression of proinflammatory molecules/mediators (cathepsin S) in RA?

3) Does anti-rheumatic treatment affect RNA editing levels?

3. Study population and Methodology

3.1 Patient recruitment and follow-up

Peripheral blood was collected in EDTA tubes (BD Vacutainer) from 19 consecutive consenting patients with RA fulfilling the 2010 ACR/EULAR criteria [202] and 14 apparently healthy controls (HC). All patients had active disease (DAS28-ESR: 3.4-7.1) at the time of sampling. Exclusion criteria included viral or bacterial infection during the past month and severe co-morbidities (cancer, heart or kidney failure). Clinical and laboratory RA features [28 tender/swollen joint count, erythrocyte sedimentation rate (ESR; mm/1st h.), C-reactive protein (CRP; mg/dl), visual analogue scale (VAS)-patient global] and rheumatoid factor (RF)/anti-cyclic citrullinated peptide (CCP) status were recorded at baseline.

RA patients were re-examined 12 weeks after initiation of new treatment (scale-up of treatment, conventional synthetic disease-modifying antirheumatic drugs (csDMARDS) ± corticosteroids and/or biological DMARDS) and were categorized in responders and moderate/non-responders according to EULAR's response criteria [205]. Treatment modalities and their effect on disease characteristics in EULAR responders vs moderate/non-responders are shown in **Table 5**. For schematic representation of study design and patient recruitment see **Figure 7**. All participants gave written informed consent in compliance with the Declaration of Helsinki, which had been previously approved by the Ethics Committee of Laiko Hospital, Athens, Greece (Protocol Nr.:1368/ 17-11-2016), as well as by the Hellenic Data Protection Authority (Protocol Nr.:ΓΝ/ΕΞ/7901-2/22-12-2016).

The EULAR response criteria

The European League Against Rheumatism (EULAR) has established certain criteria that allow Rheumatologists to evaluate therapeutic response. [205] According to DAS28 score at follow-up visit, as well as the change in DAS28 compared to previous visit, the patients are grouped in good, moderate and non-responders (see table below).

EULAR response criteria			
DAS28 at endpoint	Improvement in DAS28		
	>1.2	0.6< ΔDAS28 ≤1.2	≤0.6
≤3.2	good	moderate	none
3.2 < ΔDAS28 ≤5.1	moderate	moderate	none
>5.1	moderate	none	none

3.2 Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were isolated by ficoll density gradient centrifugation (Ficoll-Paque PLUS, GE Healthcare) within 2 hours from venipuncture. Peripheral blood collected in EDTA tubes was first diluted 1:1 with 1x PBS containing no Ca/Mg. After centrifugation at 400g for 30min. (room temperature), the upper phase containing platelet rich plasma was discarded and the PBMCs layer was transferred into a new 15ml sterile falcon tube. After two washes with PBS at 200g for 10min. each cell pellet was finally lysed in Trizol (ThermoFisher Scientific) and stored at -80ºC until further use.

3.3 RNA extraction and reverse transcription

Total RNA was isolated from patient and control PBMCs using Direct-Zol RNA Miniprep kit (Zymo research) according to manufacturer's instructions with an additional step of DNase digestion. One(1) μg of total RNA was reverse transcribed into complementary DNA (cDNA) using the MuLV reverse transcriptase kit (Invitrogen), as previously described [100].

3.4 Quantitative polymerase chain reaction

For the quantification of the two ADAR1 isoforms (ADAR1p110 and ADAR1p150) specifically designed Taqman primers were used (ADAR1p110: Hs01017596; ADAR1p150: Hs01020780, Applied Biosystems) while TATA-Box binding protein (TBP) (Hs00427621, Applied Biosystems) served as the housekeeping gene. Quantification of cathepsin S, human antigen R (HuR; *ELAVL1*) and ADAR2 (*ADARB1*) was conducted with Takara SYBR Premix Ex Taq, while RPLP0 served as housekeeping gene. The relative expression of each gene was determined according to the formula $2^{-\Delta Ct}$ ($\Delta Ct = Ct_{(gene)} - Ct_{(housekeeping\ gene)}$).

Table 4. Primers used for qPCR measurements

Gene	Forward primer	Reverse primer
RPLP0	5'-TCGACAATGGCAGCATCTAC-3'	5'-ATCCGTCTCCACAGACAAGG-3'
CTSS	5'-TCATACGATCTGGGCATGAA-3'	5'-AGGTTCTGGGCACTGAGAGA-3'
HuR (ELAVL1)	5'-GAAGACCACATGGCCGAAGA-3'	R:5'-CCAAGCTGTGTCCTGCTACT-3'
ADAR2 (ADARB1)	5'-TCCTGCAGTGACAAGATTGC-3'	5'-GTAAATGGGCTCCACGAAAA -3'

3.5 Alu Sanger sequencing and RNA editing analysis at single nucleotide level

cDNA from PBMCs was subjected to PCR with KOD Hot-start DNA polymerase (Millipore) for the amplification of the most edited *Alu* region in cathepsin S 3' untranslated region (UTR), namely *AluSx*$^{+}$ (PCR product size: 442bp). [100] Primers used for amplification of cathepsin S *AluSx*$^{+}$*:* F: 5'- GGCTCCTTCTCCATAAAGCA – 3'; R: 5' – AAAGTAGGCTGGGCTCAGTG - 3'

Gel-extracted PCR product (Zymoclean Gel DNA Recovery Kit; Zymo research) was subjected to Sanger sequencing. A-to-I RNA editing rate of individual adenosines was determined following the analysis of the chromatopherograms as we have previously described. [100]

Analysis of RNA-sequencing dataset of synovial tissues

A large RNA-sequencing dataset (GSE89408) [206] including in total 202 synovial biopsy samples (normal joint=28, osteoarthritis=22, early RA- disease duration <1 year=57, established RA=95) was accessed through the NCBI database. Aligned data on ADAR1 and cathepsin S expression were extracted through the Gene Expression Omnibus database.

3.6. Analysis of RNA-sequencing dataset for ADAR1p110 and ADAR1p150 isoform expression

RNA-sequencing data (read length 101 bp generated from total RNA) from synovial samples of normal (28), osteoarthritis (22), early rheumatoid arthritis (57) and established rheumatoid arthritis (95) patients, were downloaded from the NCBI Sequence Read Archive (SRP092408) in .sra format and converted in .fastq by means of fastqdump program that is part of the SRA toolkit package. Low-quality reads were

discarded by filtering with the NGS QC Toolkit [207] and default parameters (cutoff read length for HQ=70%, cutoff quality score=20). High quality cleaned reads were mapped against pre-indexed human genome GRCh37, transcriptome (pre-processed set of known splice junctions from Ensembl annotation), and dbSNP common release 144 using HISAT2 version 2.1.0 [208] and default parameters. Unique and concordant alignments in .sam format were converted in the binary .bam format, sorted by genomic coordinates, and indexed by SAMtools. Transcriptome quantification was performed for each sample with StringTie v1.3.6 release [209] and differential expression was tested with the DESeq2 1.24.0 [210] (FDR≤0.05) R package. Reference human transcriptome was obtained from UCSC (http://hgdownload.cse.ucsc.edu/goldenpath/hg19/database/). Expression of ADAR1 isoforms, ADAR1p150 (NM_001111) and ADAR1p110 (NM_001025107), was determined for each sample.

Bioinformatic analysis was performed in collaboration with my colleague Dr. Domenico Alessandro Silvestris, whom I want to cordially thank at this point.

3.7 Statistical analysis

Statistical analysis was conducted with SPSS 24.0. Normality of continuous variables was graphically assessed by histograms and P-P plots, as well as by Kolmogorov-Smirnov and Shapiro-Wilk tests. Pairwise differences were evaluated with two independent samples Student's t-test or the non-parametric Mann-Whitney U test for continuous variables between groups of RA and controls and Fisher’s exact chi-squared test for nominal variables. The Kruskal-Wallis test was used when comparing more than 2 groups. In order to account for multiple comparisons, Bonferroni correction was performed where applicable. Linear regression analysis was used to

control for the effect of confounding factors. Correlations between continuous variables were explored with Pearson's test or Spearman's rank test. Changes in continuous variables following treatment (same patients) were examined with the use of paired t-test or Wilcoxon signed-rank test for non-normal variables. Results were considered statistically significant when $P<0.05$.

Sample size calculation

A first interim analysis of 5 RA patients and 5 controls showed that in terms of power considerations, a total sample size of 20 subjects allocated in two equal groups would provide adequate power (i.e. over 80%) to detect a difference of 0.45 units of relative ADAR1p150 expression levels by the non-parametric Mann-Whitney test for independent samples. Measures of dispersion and anticipated differences were derived from pilot data from an interim analysis of our study (5 cases and 5 controls, mean±SD 2.104±0.363 versus 1.637±0.283, respectively). A priori power analysis was performed with G*Power v 3.1.9.4. [211]

Table 5: Demographics, clinical and laboratory features of controls and RA patients at baseline and after 12-week treatment

	Rheumatoid Arthritis (n=19)	EULAR Responders (n=11)		EULAR moderate/ non-responders (n=8)		Controls (n=14)
	Baseline	**Baseline**	**12 weeks**	**Baseline**	**12 weeks**	**Baseline**
Men/women, n	4/15	2/9		2/6		5/9
Age (mean±SD, years)	54.2±16.0	47.9±14.8		62.8±14.1		39.0 ± 12.6
Disease duration (mean±SD, years)	7.3±7.4	6.0±7.2		9.0±7.6		n/a
Disease activity						
Tender joint count (28), n	7.9±5.3	7.3±5.4	1.0±1.8*	8.8±5.5	5.4±4.7*	0
Swollen joint count (28), n	5.4±4.0	3.9±3.8	0.8±1.5*	7.5±3.6	4.0±3.2*	0
ESR (mm/1st h)	34±23	33±28	12±15*	36±15	26±7*	n/a
CRP (mg/dl)	1.7±1.5	1.6±1.9	0.3±0.3*	1.9±0.9	0.9±0.6*	n/a
DAS28-ESR	5.2±1.2	5.0±1.3	2.0±0.8*	5.5±1.1	4.5±1.2*	n/a
Auto-antibodies						
RF positivity, n (%)	11 (57.9)	6 (54.5)		5 (62.5)		n/a
anti-CCP positivity, n (%)	12 (63.2)	7 (63.6)		5 (62.5)		n/a
Therapy						
csDMARDs, n (%)	5 (26.3)	3 (27.3)	6 (54.5)	2 (25.0)	4 (50.0)	0
corticosteroids, n (%)	6 (31.6)	3 (27.3)	5 (45.5)	3 (37.5)	4 (50.0)	0
anti-TNF, n (%)	0 (0.0)	0 (0.0)	6 (54.5)	0 (0.0)	5 (62.5)	0
anti-IL-1, n (%)	0 (0.0)	0 (0.0)	2 (18.2)	0 (0.0)	0 (0.0)	0
anti-CD20, n (%)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (12.5)	0

ESR: erythrocyte sedimentation rate; *CRP*: C-reactive protein; *DAS*: disease activity score; *RF*: rheumatoid factor; *anti-CCP*: anti-cyclic citrullinated peptide; *csDMARDS*: conventional synthetic disease-modifying antirheumatic drugs(methotrexate, leflunomide); *TNF*: tumor necrosis factor; *IL-1*: interleukin 1; *P<0.05 compared to baseline

Figure 7

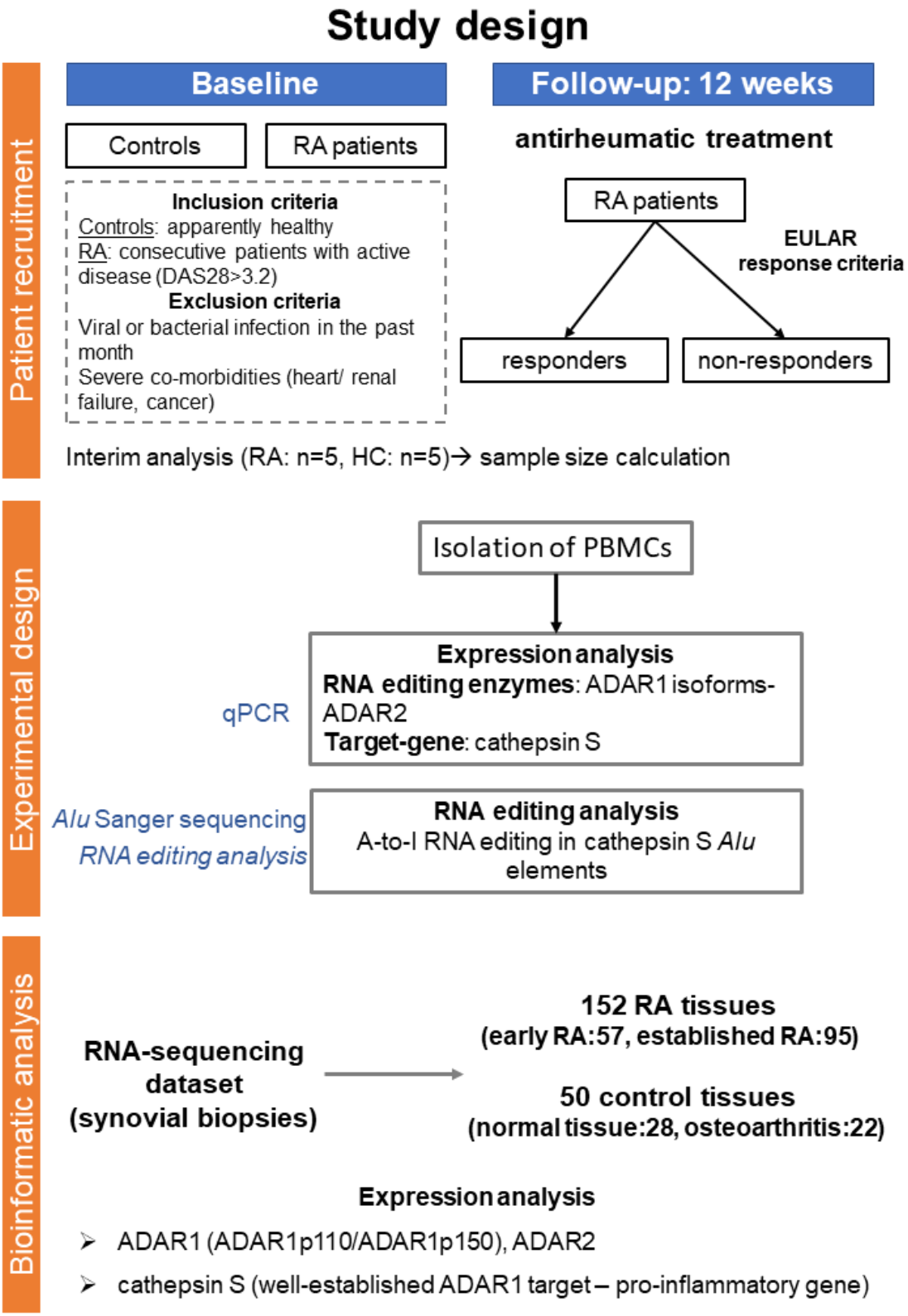

Figure 7. Schematic representation of the study design

4. Results

4.1 Increased ADAR1p150 expression in peripheral blood and synovium of RA patients

4.1.1 The RNA editor ADAR1 is increased in RA synovium

Expression analysis of the RNA-sequencing dataset GSE89408 revealed significant upregulation of total ADAR1 in synovium of patients with either early RA or established RA (P<0.001 for each comparison: early or established RA vs. normal synovium or osteoarthritis, **Figure 8A**). Interestingly there was a trend upregulation of ADAR1 in early RA when compared to established RA (P=0.06 early vs. established RA). The expression levels of the second RNA editor ADAR2 (*ADARB1*) were similar in RA patients and controls (**Figure 8B**).

Figure 8

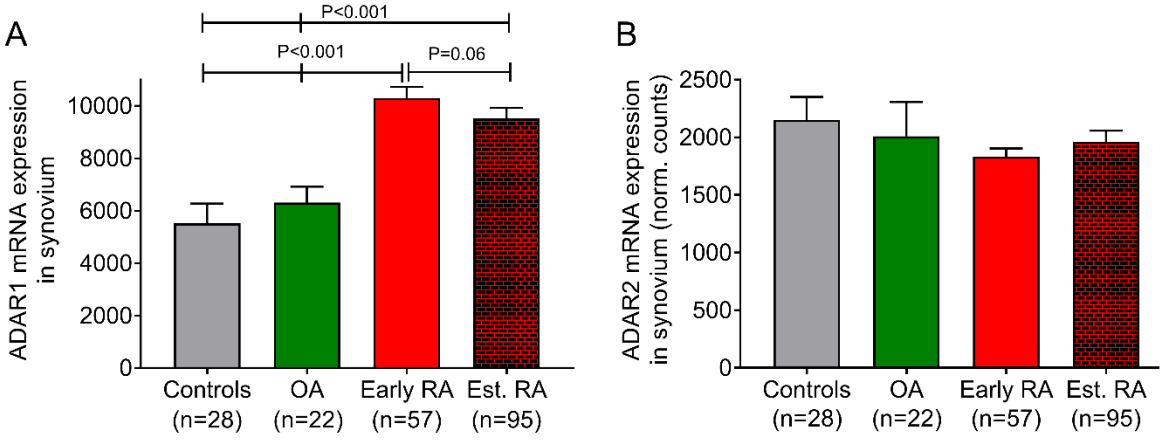

Figure 8. Total ADAR1 and ADAR2 expression in synovium. *Bar-graphs showing total ADAR1 (**A**) and ADAR2 (**B**) mRNA expression levels in the synovium of patients with early RA (disease duration<1 year), established RA (est. RA), osteoarthritis (OA) or healthy synovium (controls). P-values are derived from Mann-Whitney U test. Bar graphs represent mean+SEM (standard error mean).*

4.1.2 ADAR1p150 isoform but not ADAR1p110 is increased in RA synovium

ADAR1 has two isoforms, namely ADAR1p110 and ADAR1p150. [15,16] ADAR1p110 is constitutively expressed, [16] while the interferon-inducible ADAR1p150 has also been reported to be induced by the proinflammatory cytokines TNFα and interferon-γ [100] . Our analysis showed that expression of ADAR1p150 isoform was significantly increased in rheumatoid synovium (**Figure 9A**), whereas mRNA levels of the constitutively expressed ADAR1p110 isoform (**Figure 9B**) did not differ between diseased and control synovium.

Figure 9

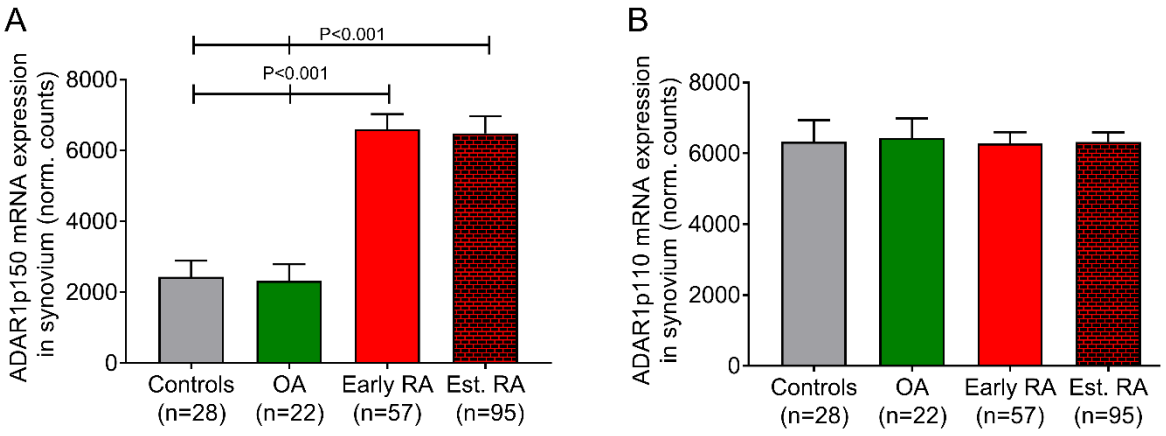

Figure 9. ADAR1 isoform expression in RA synovium. *Bar-graphs (mean+SEM) representing ADAR1p150* ***(A)*** *and ADAR1p110* ***(B)*** *mRNA expression in synovial tissue of controls, osteoarthritis, early RA (disease duration<1 year) and established RA patients. P-values are derived from Mann-Whitney test. Abbreviations: OA: osteoarthritis; Est. RA: established RA; SEM: standard error of mean.*

4.1.3 Increased ADAR1p150 expression in PBMCs of active RA

Similarly, ADAR1p150 isoform was significantly increased in PBMCs of active RA patients (**Figure 10A**) , while no significant difference in ADAR1p110 levels (**Figure 10B**) or the second RNA editor, ADAR2 (**Figure 10C**), were observed.

Figure 10

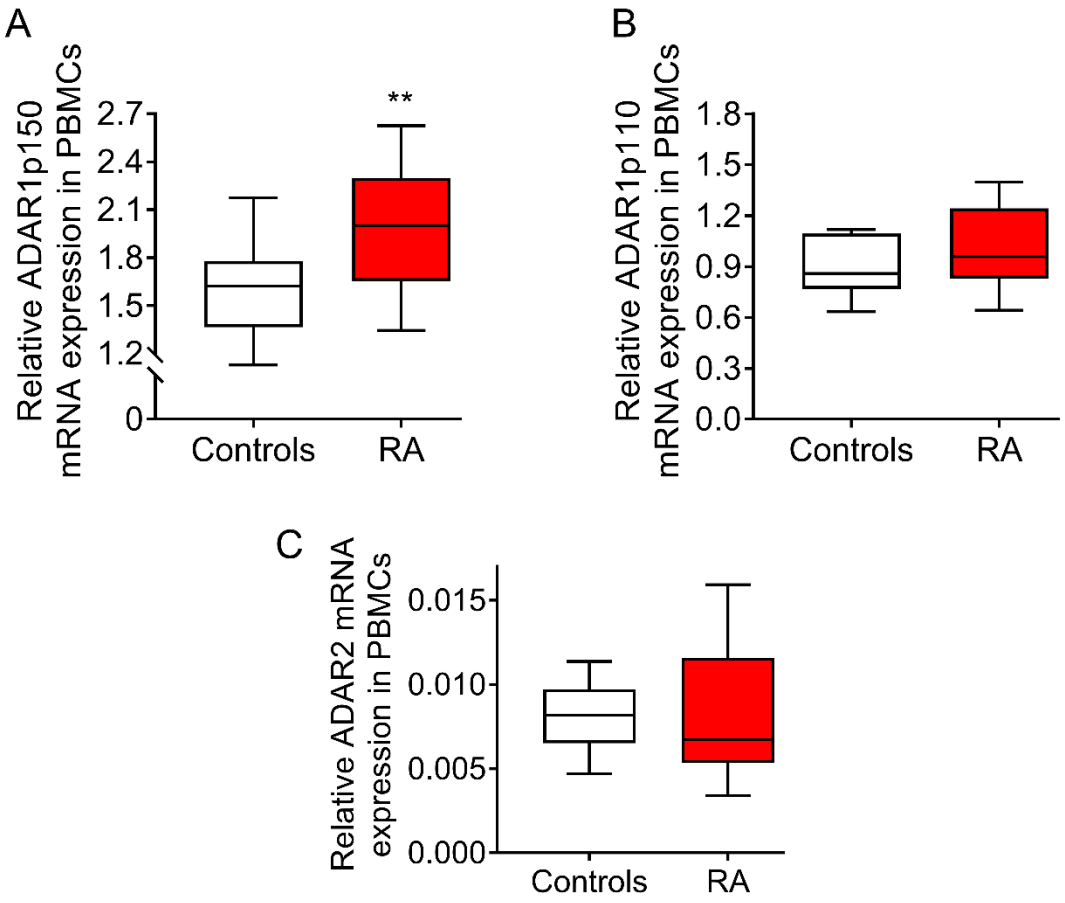

Figure 10. ADAR1 isoforms and ADAR2 mRNA expression in active RA PBMCs. Tukey box-plots showing *mRNA expression levels of the interferon-inducible ADAR1p150 isoform (**A**) and the constitutively expressed ADAR1p110 isoform (**B**), as well as of the second RNA editor ADAR2 (**C**) in PBMCs of patients with active RA (n=19) and controls (n=14) as determined by qPCR with specifically designed Taqman primers (ADAR1p110: Hs01017596 ; ADAR1p150: Hs01020780, Applied Biosystems) or SYBR-Green PCR (ADAR2). Expression*

*levels were normalized with the use of TBP as housekeeping gene for TaqMan expression assays (Hs00427621, Applied Biosystems) and RPLP0 for SYBR-Green qPCR. **P<0.01*

Taken together, these results suggest that ADAR1, and particularly its inducible isoform ADAR1p150, is increased in RA in both the target-tissue (synovium) and in the peripheral blood (PBMCs), indicating that ADAR1p150 is the only RNA editing enzyme induced under proinflammatory conditions in RA patients.

4.2 Increased Alu RNA editing rate in active RA

In order to evaluate the RNA editing activity of ADAR1, we sequenced the *AluSx*$^{+}$ region that is located in cathepsin S mRNA 3' UTR (**Figure 11**), which is edited only by ADAR1 and is responsible for the post-transcriptional regulation of cathepsin S mRNA stability and expression. [100] Since inosines are recognized by the reverse transcriptase as guanosines, we could detect A-to-I RNA editing levels of modified adenosines as A-to-G nucleotide mismatches when we compared the RNA sequence with the genome.

Figure 11

Figure 11. *Schematic representation of cathepsin S 3' UTR and cDNA sequence of cathepsin S AluSx*$^{+}$*. All detected edited adenosines are depicted in green, while individual adenosines with significantly increased RNA editing rates in active RA PBMCs are depicted in red. HuR binding sites are underlined.*

We were able to detect more than 20 edited adenosines in cathepsin S *AluSx*$^{+}$ (**Table 6**), 8 of which had significantly higher editing rates in PBMCs of active RA patients compared to controls (6-47% mean increase in editing rate of 8 individual adenosines, all $P<0.05$; **Figure 12**).

Figure 12

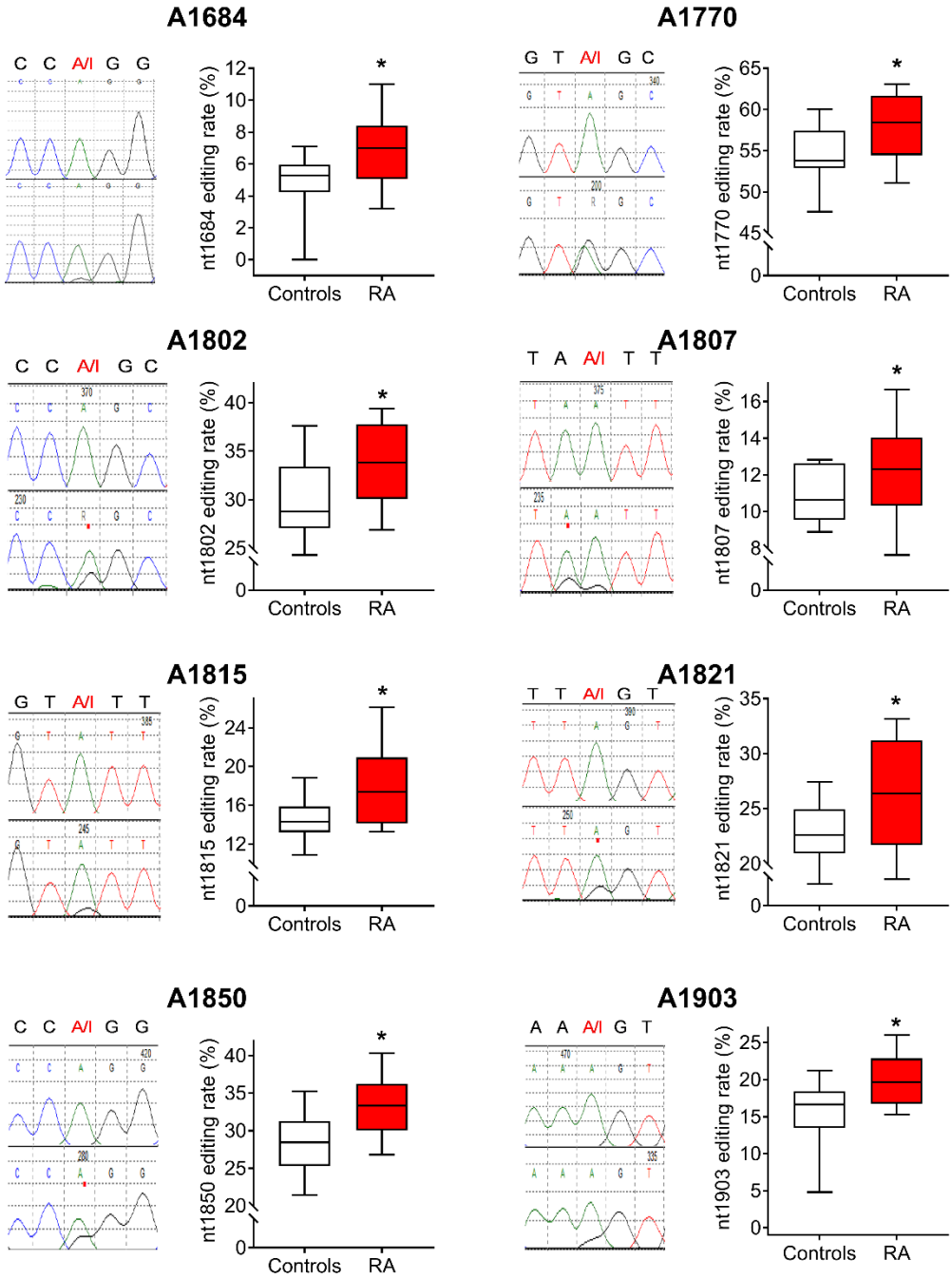

Figure 12. *Chromatopherograms and boxplots depict RNA editing rate of 8 individual adenosines in PBMCs of active RA patients (n=19) vs healthy controls (n=14). RNA editing rates of individual adenosines were determined following analysis of chromatopherograms. *P<0.05 by Mann-Whitney test or independent samples t-test.*

Both the average of all edited adenosines (P=0.02) in *AluSx*$^+$ as well as the average RNA editing rate of the 8 significantly higher edited nucleotides (from now on referred to as mean cathepsin S *AluSx*$^+$; P=0.003, **Figure 13A**) were significantly higher in active RA PBMCs. Mean cathepsin S *AluSx*$^+$ RNA editing rate correlated with the expression of the inducible ADAR1p150 isoform (n=19, r=0.623, P=0.004, **Figure 13B**), but not with the constitutively expressed ADAR1p110 isoform (r=0.266, P=0.271, **Figure 13C**), further supporting the role of ADAR1p150 as the main RNA editor in RA.

Figure 13

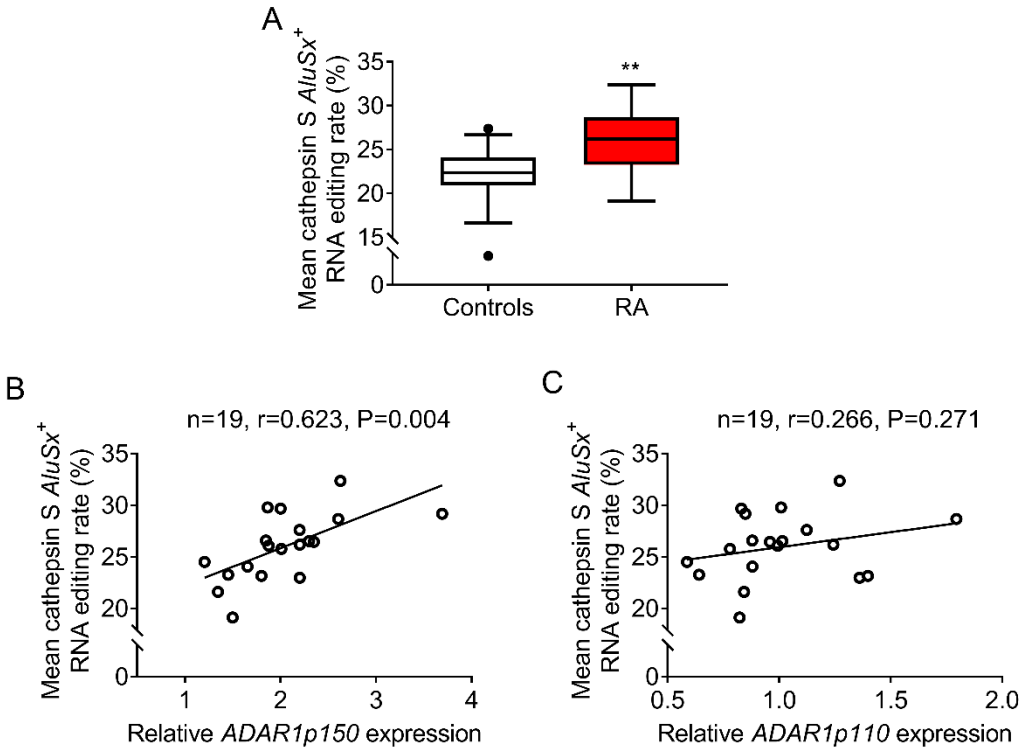

Figure 13. A. *Boxplot of mean cathepsin S AluSx*$^+$ *RNA editing rate of the 8 edited adenosines in PBMCs from patients with active RA (n=19) vs. controls (n=14) and scatter-plot showing correlation of individual RNA editing rates with ADAR1p150 (**B**) or ADAR1p110 (**C**) mRNA levels in RA patients. **P<0.01 by Mann-Whitney test.*

Moreover, the RNA editing rates of 16 individual adenosines within cathepsin S 3' UTR *AluSx*$^{+}$ strongly correlated with ADAR1p150 mRNA levels (r= range 0.454-0.754, P≤0.05 for all- **Table 6)**.

Table 6. Correlation of RNA editing rate of individual adenosines located in cathepsin S 3' UTR *AluSx*$^{+}$ with ADAR1p150/ ADAR1p110 and cathepsin S mRNA expression in RA PBMCs

	ADAR1p150	**ADAR1p110**	**Cathepsin S**
A1662	**r=0.598 (P=0.007)**	**r=0.546 (P=0.02)**	r=0.249 (P=0.30)
A1672	**r=0.463 (P<0.05)**	**r=0.512 (P=0.03)**	r=0.446 (P=0.06)
A1684	**r=0.740 (P<0.001)**	r=0.405 (P=0.09)	**r=0.498 (P=0.03)**
A1710	**r=0.611 (P=0.005)**	r=0.325 (P=0.18)	r=0.318 (P=0.19)
A1735	**r=0.619 (P=0.005)**	r=0.423 (P=0.07)	r=0.449 (P>0.05)
A1758	**r=0.707 (P=0.001)**	**r=0.518 (P=0.02)**	**r=0.472 (P=0.04)**
A1770	**r=0.700 (P=0.001)**	**r=0.495 (P=0.03)**	**r=0.558 (P=0.01)**
A1777	r=0.202 (P=0.41)	r=-0.08 (P=0.74)	r=0.089 (P=0.72)
A1780	**r=0.616 (P=0.005)**	r=0.386 (P=0.10)	r=0.400 (P=0.09)
A1802	**r=0.521 (P=0.02)**	r=0.325 (P=0.18)	**r=0.537 (P=0.02)**
A1806	**r=0.670 (P=0.002)**	r=0.393 (P=0.10)	**r=0.632 (P=0.004)**
A1807	**r=0.595 (P=0.007)**	r=0.344 (P=0.15)	**r=0.540 (P=0.02)**
A1815	**r=0.647 (P=0.003)**	r=0.388 (P=0.10)	r=0.437 (P=0.06)
A1821	**r=0.754 (P<0.001)**	**r=0.493 (P=0.03)**	**r=0.581 (P=0.009)**
A1824	**r=0.454 (P=0.05)**	r=0.263 (P=0.28)	**r=0.716 (P=0.001)**
A1825	r=0.440 (P=0.06)	r=0.254 (P=0.29)	**r=0.533 (P=0.02)**
A1826	**r=0.728 (P<0.001)**	r=0.446 (P=0.06)	**r=0.558 (P=0.01)**
A1830	r=0.388 (P=0.10)	r=0.188 (P=0.44)	r=0.284 (P=0.24)
A1843	r=0.314 (P=0.19)	r=0.005 (P=0.98)	r=0.209 (P=0.39)
A1846	r=0.435 (P=0.06)	r=0.186 (P=0.45)	**r=0.461 (P<0.05)**
A1850	r=0.328 (P=0.17)	r=0.018 (P=0.94)	r=0.346 (P=0.15)
A1888	r=-0.019 (P=0.94)	r=-0.188 (P=0.44)	r=0.000 (P=1.00)
A1903	r=0.140 (P=0.57)	r=0.132 (P=0.59)	r=0.260 (P=0.28)
A1909	**r=0.616 (P=0.005)**	**r=0.465 (P<0.05)**	**r=0.628 (P=0.004)**

A signifies the nucleotide position of each adenosine in cathepsin S 3' UTR. Correlations were examined with Spearman's rank test. Statistical significance was set at P=0.05

4.3 The effect of antirheumatic treatment on RNA editing

Next, we investigated whether the observed upregulation of ADAR1p150 and *AluSx*$^{+}$ RNA editing rate in active RA was reversible after 12 weeks of antirheumatic treatment with csDMARDS, corticosteroids and/or bDMARDs. Of note, both ADAR1p150 expression levels and average *AluSx*$^{+}$ RNA editing rate decreased post-treatment only in EULAR responders (P=0.008 and P=0.02, respectively) (**Figure 14A,B**), while they remained unaffected in those patients with moderate to no response (**Figure 14C,D**). These results indicate that the effect of the antirheumatic treatment on RNA editing machinery depends on the clinical response to the given therapy, thus suggesting that the dynamic regulation of *Alu* A-to-I RNA editing reflects the course of RA.

Figure 14

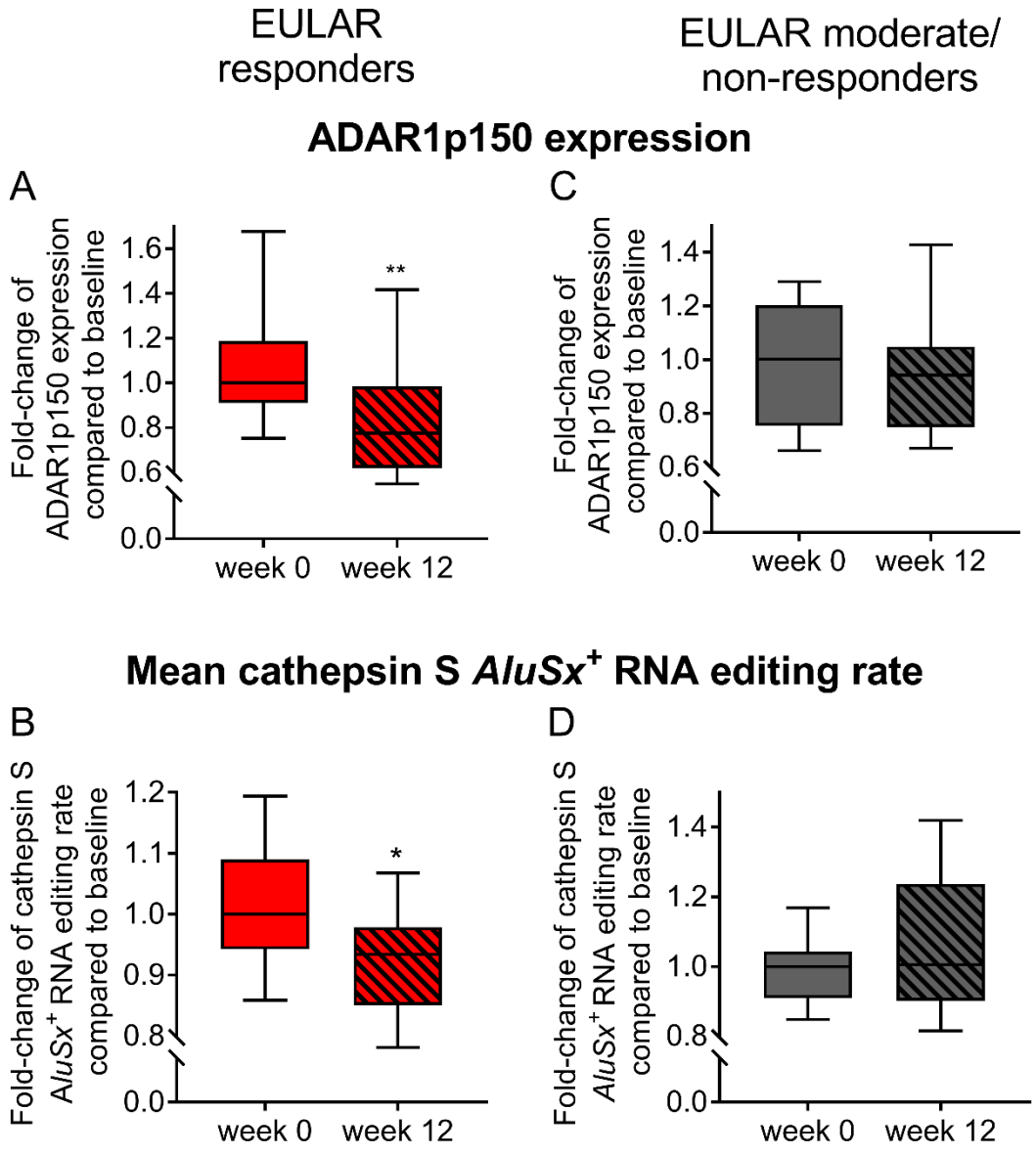

Figure 14. Effect of antirheumatic treatment on ADAR1p150 and RNA editing is dependent on clinical response. *The effect of antirheumatic treatment on ADAR1p150 mRNA expression levels and cathepsin S AluSx$^+$ RNA editing rate at baseline (week 0) and after 12-week treatment in EULAR responders (**A,B**) and moderate/non-responders (**C,D**). Individual ADAR1p150 expression levels and RNA editing rate of cathepsin S AluSx$^+$ were standardized to the baseline median per group to show the effect of treatment (fold-change). *P<0.05; **P<0.01 by Wilcoxon's signed-rank test or paired t-test.*

4.4 The effect of A-to-I RNA editing on proinflammatory gene expression in RA

A-to-I RNA editing of *Alu* elements disrupts the double-stranded RNA structure through the production of weak I-U bonds, unwinding double-stranded RNA secondary structure [19]. In this way, double-stranded RNA is regionally converted into a more single-stranded structure enabling the binding of single-strand RNA-binding proteins, such as HuR (*ELAVL1*), which can stabilize mRNAs and, thus, increase their expression, as we have previously described for cathepsin S mRNA. [100]

Herein, we used cathepsin S, not only as a well-established target of ADAR1 [100], but also as an important molecule in RA development [212], to test whether the observed upregulation of ADAR1-mediated *Alu* RNA editing rate was associated with increased cathepsin S mRNA expression in RA. First, we confirmed previous reports showcasing that cathepsin S mRNA expression was significantly increased in PBMCs of active RA patients (1.34-fold increase compared to healthy controls, $P<0.05$, **Figure 15A**), as well as at the synovium of RA patients (6-fold increase compared to normal synovium, $P<0.001$, **Figure 15B**). More importantly, cathepsin S expression significantly correlated with the proinflammatory ADAR1p150 isoform ($r=0.623$, $P=0.004$), as well as with the RNA editing rate of 12 individual adenosines within cathepsin S *AluSx*$^{+}$ (r=range 0.461-0.716, $P\leq0.05$ for all; **Table 6**) and mean RNA editing rate ($r=0.589$, $P=0.008$, **Figure 15C**) in active RA. In contrast, no association was observed between the constitutively expressed ADAR1p110 isoform and cathepsin S mRNA levels ($r=0.268$, $p=0.27$; data not shown). Next, we studied the association between the expression of ADAR1 and cathepsin S in a large cohort of RA synovial tissue. In line with the results in the PBMCs, a correlation between ADAR1 and cathepsin S mRNA was also observed in synovial tissue derived from RA patients

(n=152, r=0.516, P<0.001, **Figure 15D**) indicating that inflammation-induced *Alu* A-to-I RNA editing may augment cathepsin S mRNA.

Figure 15

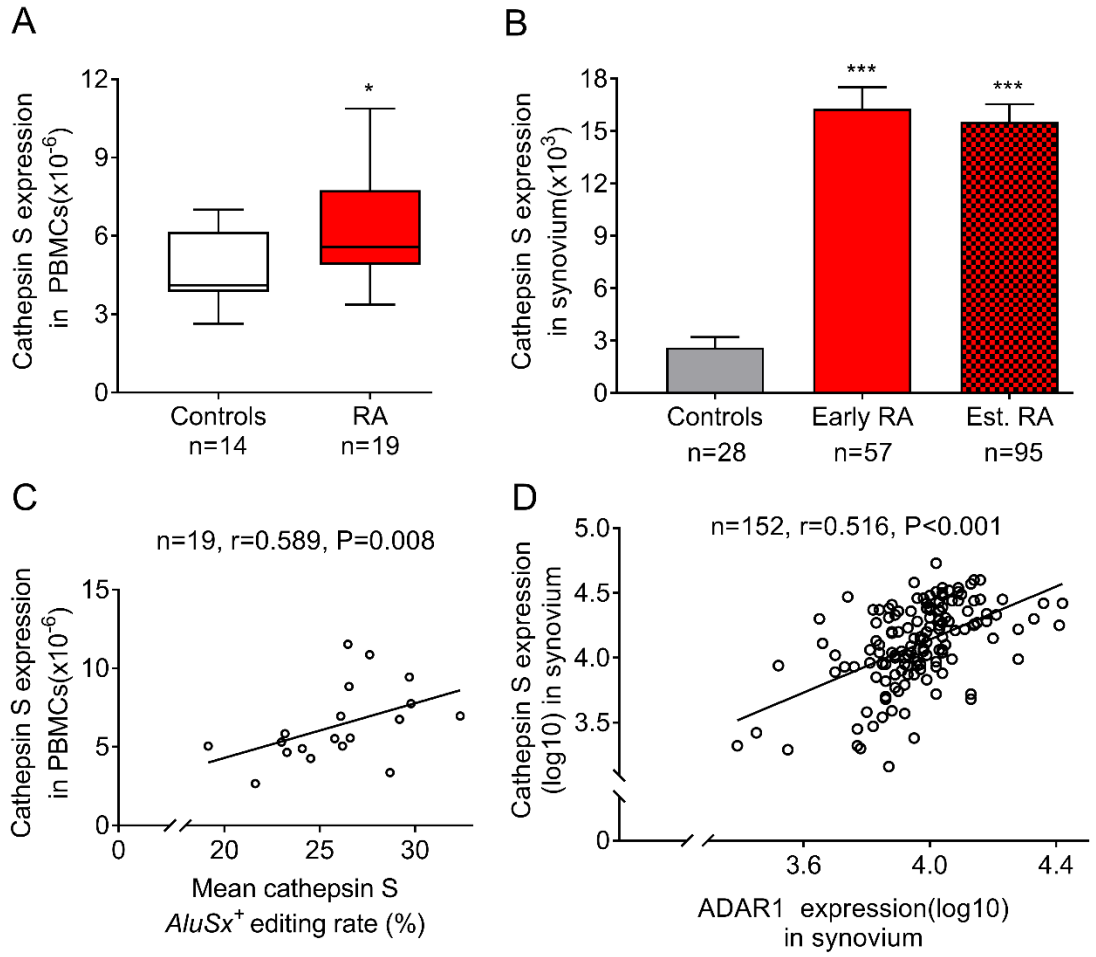

Figure 15. Cathepsin S mRNA expression in RA ***A.*** *Cathepsin S mRNA expression in PBMCs of patients with active RA and controls were quantified by RT-qPCR.* ***B.*** *Expression analysis of cathepsin S mRNA levels at synovial biopsies derived from the RNA sequencing dataset GSE89408. Aligned RNA-seq. data were downloaded from Gene Expression Omnibus.* ***C.*** *Scatterplot showing correlation of individual cathepsin S mRNA levels with RNA editing rate in active RA PBMCs.* ***D.*** *Scatterplot showing correlation of ADAR1 and cathepsin S mRNA levels at synovial tissue of RA patients from RNA-seq. dataset GSE89408. *P<0.05; ***P<0.001 by Mann-Whitney test.*

Correlation of cathepsin S with HuR in RA PBMCs

Since RNA editing controls cathepsin S expression through increased HuR binding to *AluSx*$^+$, [100] we statistically controlled for the potential involvement of HuR in regulation of cathepsin S expression by ADAR1p150-mediated RNA editing in RA. Towards this goal, we used a linear regression analysis for cathepsin S mRNA expression levels (dependent variable) and ADAR1p150 or average cathepsin S *AluSx*$^+$ RNA editing rate controlling for the effect of HuR expression in these samples. Indeed, controlling for HuR abolished the observed relationship between cathepsin S mRNA expression and ADAR1p150 (P=0.58) or average *AluSx*$^+$ RNA editing (P=0.29) suggesting that regulation of cathepsin S expression by RNA editing in RA is mainly mediated by HuR. In support of this notion, HuR expression significantly correlated with cathepsin S in active RA PBMCs (r=0.629, P=0.004; **Figure 16**).

Figure 16

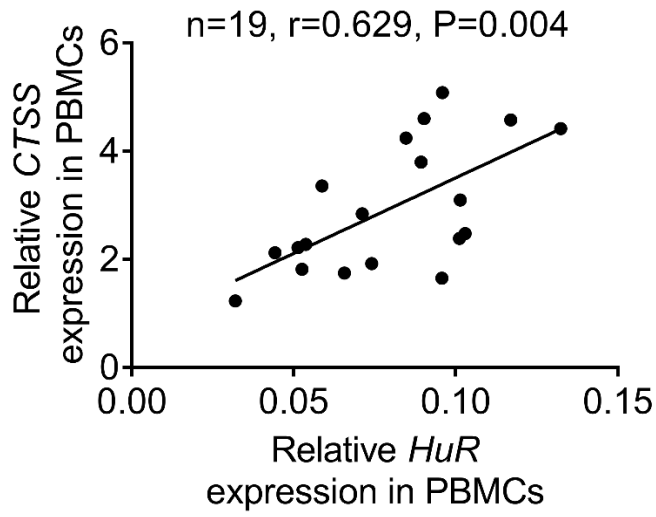

Figure 16. *Scatter-plot showing the correlation between HuR and cathepsin S (CTSS) expression in PBMCs of patients with active RA.*

Additional Alu-enriched genes possibly regulated by A-to-I RNA editing in RA

Of note, cathepsin S is only an example among several *Alu*-enriched proinflammatory genes, which are predicted to be highly edited according to the RNA editing database RADAR [213] and whose regulation by RNA editing in inflammatory disease warrants further investigation. For example, TNF receptor-associated factors TRAF1, TRAF2, TRAF3, TRAF5 have 18-1,135 predicted RNA editing sites in their *Alu* elements. Of interest, when we analysed their expression in rheumatoid synovium, we found a significant correlation with ADAR1 expression (n=152, r= range 0.356-0.743, **Table 7**).

Table 7. *Alu*-enriched molecules involved in TNF-signaling pathway predicted to be edited

	Number of predicted editing sites in *Alus*	**Correlation with ADAR1 in RNA-seq.** (GSE89408, n=152)
TRAF1	18	**r=0.508 (P<0.001)**
TRAF2	292	**r=0.732 (P<0.001)**
TRAF3	1135	**r=0.743 (P<0.001)**
TRAF5	123	**r=0.356 (P<0.001)**
RIPK1	119	**r=0.637 (P<0.001)**

Number of predicted A-to-I RNA editing sites was extracted from RADAR RNA editing database (http://rnaedit.com). Correlation of Alu-enriched genes with ADAR1 was examined in the RA samples of RNA-seq. dataset GSE89408 using Spearman's rank test.

Taken together, our findings imply that *Alu* RNA editing may be a global primate-specific mechanism controlling the expression of *Alu*-enriched inflammatory mediators at post-transcriptional level through HuR-mediated RNA processing/stability in patients with RA. The proposed mechanism is summarized in **Figure 17**.

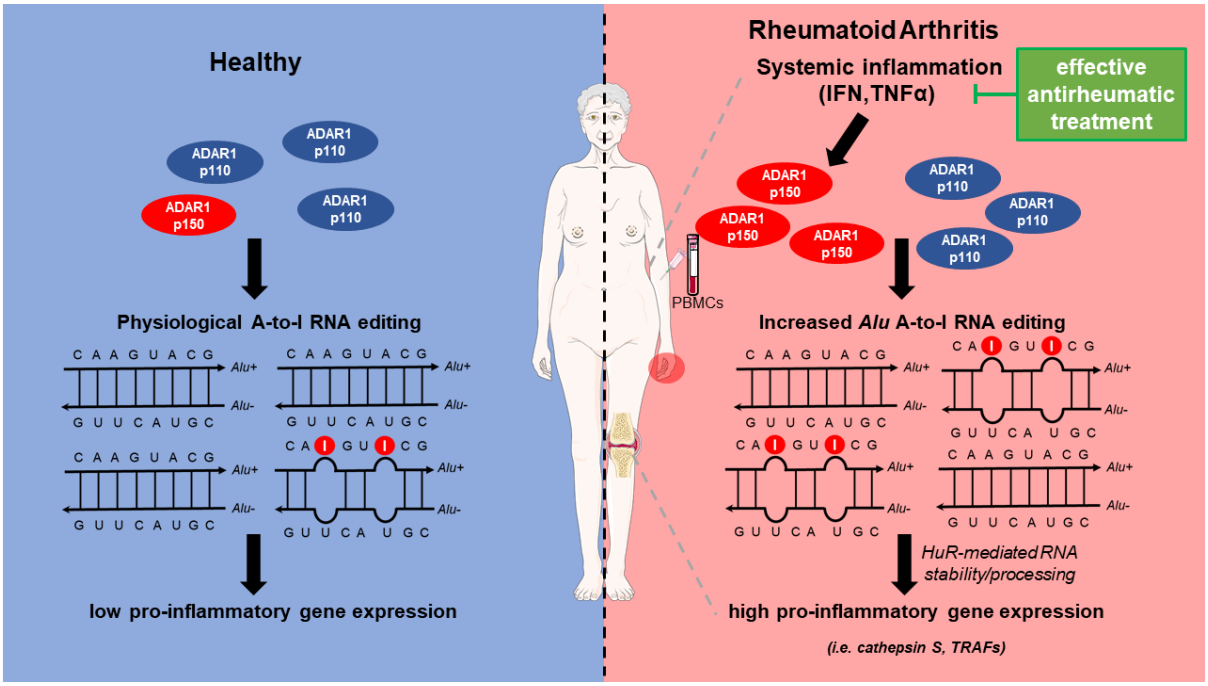

Figure 17. Proposed mechanism: Increased Alu A-to-I RNA editing fuels inflammatory gene expression in RA through post-transcriptional regulation of RNA metabolism.

Synovial or systemic inflammation induces the expression of the proinflammatory inducible ADAR1p150 isoform and consequently A-to-I RNA editing of Alu elements. Increased Alu RNA editing may fuel inflammation by controlling the expression of inflammatory mediators through post-transcriptional HuR-dependent mRNA stability/processing. Certain items on this figure have been adapted from Servier Medical Art by Servier (https://smart.servier.com – licensed under Creative Commons Attribution 3.0 Unported License), Abbreviations: IFN: interferon, HuR: human antigen R, TRAFs: TNF receptor-associated factors.

5. Discussion

The present study provides first evidence that: 1) the RNA editor ADAR1, and specifically the ADAR1p150 isoform, is increased in diseased synovium, the target tissue in RA, 2) the proinflammatory ADAR1p150 isoform as well as the A-to-I RNA editing rates of repetitive *Alu* elements are increased in PBMCs of patients with active RA and 3) after a 12-week antirheumatic treatment reduction of ADAR1p150 expression and *Alu* A-to-I RNA editing rate are prominent only in patients with good clinical response; 4) the increased *Alu* RNA editing rate is associated with increased proinflammatory gene expression in RA.

ADAR1-mediated RNA editing is indispensable for life, as mice lacking ADAR1 or having an editing-deficient knock-in mutation die *in utero.* [30,37,43] In humans, mutations in ADAR1 cause Aicardi-Goutières Syndrome associated with a type I interferon signature. [136] Further, ADAR1 and especially its isoform ADAR1p150 is a type I interferon-inducible gene. [15] Accordingly, ADAR1 expression has been found increased in type I interferon-associated autoimmune diseases, [143,144,146–149] but also in other inflammatory diseases including acute myocardial infarction, atherosclerosis, cancer and viral infections. [49,100,214] More importantly, ADAR1 expression and activity are increased after stimulation with TNFα, [100] the major cytokine that regulates the dynamics of transcriptome in RA, [215] due to a significant increase in levels of the ADAR1p150 isoform. [100] Our results herein suggest that ADAR1 and specifically the ADAR1p150 isoform is increased in RA through a synergistic effect driven by TNF and type I interferon signaling. The upregulation of ADAR1 was found at the inflammatory tissues (synovium) as well as in the circulation, and, therefore, ADAR1 may have an impact on other autoimmune diseases besides

RA. Further studies are warranted to elucidate the systematic and tissue- and cell-specific regulation and role of ADAR1, and more specifically of the proinflammatory ADAR1p150 isoform, in systemic autoimmunity.

ADAR1-induced A-to-I RNA editing takes place primarily in repetitive *Alu* elements, therefore comprising an upstream regulatory mechanism of RNA metabolism specific for primates. [14,19,21,22,104] The widespread *Alu* elements have recently emerged as critical regulators of inflammation [204] and an enrichment of *Alu* elements has been recently reported in autoimmune diseases, potentially contributing to type I interferon pathway activation. [203,204] Data in systemic autoimmune disease are yet controversial, as studies have shown up-regulation of global *Alu* RNA editing index in peripheral blood of patients with systemic lupus erythematosus, [148] but downregulation in keratinocytes of patients with psoriasis. [149] Clearly, comparative studies at transcriptome-wide level and especially at a single nucleotide or *Alu* level of individual transcripts in inflammatory and autoimmune diseases are warranted to enlighten the tissue-specific and disease-specific effects of *Alu* A-to-I RNA editing. The difference between global and transcript-specific RNA editing levels among the inflammatory and especially the autoimmune diseases may reflect not only the tissue-specificity of RNA editing, [150] but also the inflammatory microenvironment and milieu in each disease. [216] Moreover, we found that effective anti-inflammatory treatment was able to decrease RNA editing levels in our patients. To the best of our knowledge this is the first report showing the effect of anti-inflammatory treatment on RNA editing levels in association with clinical response. Whether RNA editing is a predictive biomarker of clinical response and/or involved in the response to therapy remains to be investigated in future studies.

Alu RNA editing can control various aspects of RNA metabolism including splicing and mRNA stability, which can ultimately affect expression levels of edited genes. [14,19,217] One of the major mechanisms leading to increased mRNA stability is the binding of the stabilizing RNA-binding protein HuR to its target motifs TTTTG, TTTTT and ATTTA located within the *Alu* elements. [217,218] Therefore, we also checked whether increased RNA editing observed in active RA patients led to aberrant gene expression, serving probably as an additional, primate-specific regulatory mechanism at the post-transcriptional level. Towards this goal, we used as exemplar the well-established ADAR1-target cathepsin S. [100] Cathepsin S belongs to the family of lysosomal cysteine proteases, which are critically involved in antigen presentation and immune response. [219] In mice, knockout of cathepsin S prevents collagen-induced arthritis, [212] whereas increased circulating cathepsin S protein levels have also been detected in RA patients. [220] Moreover, cathepsin S may contribute to autoantibody production since it specifically mediates degradation of the invariant chain Ii, thereby promoting MHC-II antigen binding. [221,222] Previous studies have also shown a significant upregulation of cathepsin S in RA synovial fluid, [223] suggesting a role in synovial inflammation by its elastolytic properties and/or by enhancing antigen presentation and autoantibody production.

Our findings show a significant increase of cathepsin S both at the synovium and PBMCs of patients with RA which is significantly associated with the expression of ADAR1 and especially of ADAR1p150, as well as with the individual RA editing rate of the adenosines located within the *AluSx*$^{+}$ of cathepsin S 3' UTR. These associations imply that the disruption of *AluSx*$^{+}$ double-stranded RNA structure to a more single-stranded one by A-to-I RNA editing reveals the HuR target-sequences enabling the single-strand RNA-binding protein HuR to bind to its target cathepsin S,

thus increasing its mRNA stability and expression, as previously evidenced in a series of mechanistic studies. [100] Of interest, cathepsin S is only one of the many edited *Alu*-enriched genes, e.g. TRAFs, whose expression was found to be associated with ADAR1 in a large cohort of RA patients, suggesting that our proposed mechanism (**Figure 17**) may be applicable to a large number of proinflammatory genes in RA or other inflammatory diseases. Further studies are warranted to evaluate the role of A-to-I RNA editing in transcriptome metabolism and cellular function in RA.

Future perspectives

Certain points of interest remain to be addressed in the future by us and other research groups aiming to delineate the role of A-to-I RNA editing in chronic inflammatory diseases. First, what is the role of ADAR1p110 and ADAR2 in chronic inflammation? Is the observed upregulation of ADAR1-induced A-to-I RNA editing tissue- or cell-specific? The emergence of single-cell RNA-seq., which is becoming increasingly available, will help us identify specific cell subpopulations with unique transcriptional and “epi-transcriptional” profiles. Moreover, is increased RNA editing a shared mechanism among systemic autoimmune diseases? Do various proinflammatory pathways (NF-kB / type I IFN) lead to discrete changes in A-to-I RNA editing events? Similarly, how can different anti-inflammatory therapies affect RNA editing levels and do changes in RNA editing have a predictive role for therapy response? Finally, what is the role of RNA editing in the inflammatory milieu of various chronic inflammatory disorders? Does it act as a counteracting mechanism aiming to suppress excessive inflammation or is it “fuel on fire”?

6. Conclusion

To conclude, our data reveal a previously unrecognized dynamic regulation of *Alu* A-to-I RNA editing in RA that underpins therapeutic response and fuels inflammatory gene expression. The proinflammatory, inducible ADAR1p150 isoform acts as a transcriptome-wide regulator of human-specific *Alu*-enriched inflammatory gene expression and, thus, may comprise an interesting predictive biomarker and a potential therapeutic target in patients with chronic inflammatory (auto)immune-mediated diseases, as supported by recent promising results in preclinical cancer models. [97,98]

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