HELLENIC REPUBLIC
National and Kapodistrian
University of Athens
EST. 1837

SCHOOL OF MEDICINE

1st Department of Propaedeutic and Internal Medicine

Director: Petros P. Sfikakis

Oxidative stress and the DNA damage Response/Repair network in Systemic Autoimmune Diseases

(Το οξειδωτικό στρες και το δίκτυο κυτταρικής απόκρισης στην βλάβη του DNA σε ασθενείς με συστηματικά αυτοάνοσα νοσήματα)

Panagiotis Ioannis A. Ntouros, MD

Doctoral dissertation

Athens, 2024

Η έγκριση της διδακτορικής διατριβής από την Ιατρική σχολή του Πανεπιστημίου Αθηνών δεν αποτελεί παραδοχή γνωμών του συγγραφέως N5343/32 άρθρο 202, παράγραφος 2.

Στοιχεία Διδακτορική Διατριβής

Ημερομηνία αίτησης: 11/01/2019

Ημερομηνία ορισμού 3-μελούς Συμβουλευτικής Επιτροπής: 22/02/2019

Μέλη 3-μελούς Συμβουλευτικής Επιτροπής:
Καθηγητής Πέτρος Π. Σφηκάκης (Επιβλέπων)
Καθηγητής Γοργούλης Βασίλειος
Ερευνητής Α' Σουλιώτης Βασίλειος

Ημερομηνία ορισμού του Θέματος: 08/07/2019

Ημερομηνία καταθέσεως της διδακτορικής διατριβής: 22/1/2024

Επταμελής εξεταστική επιτροπή (Ορισμός 25/1/2024):
1. Σφηκάκης Πέτρος
2. Γοργούλης Βασίλειος
3. Σουλιώτης Βασίλειος
4. Τεκτονίδου Μαρία
5. Φραγκούλης Γεώργιος
6. Μαυραγάνη Κλειώ
7. Λυμπερόπουλος Ευάγγελος

Ημερομηνία υποστηρίξεως της διδακτορικής διατριβής: 28/2/2024

Βαθμολογία διδακτορικής διατριβής: ΑΡΙΣΤΑ

Ευχαριστίες

Η παρούσα διδακτορική διατριβή εκπονήθηκε στην Ρευματολογική μονάδα και το εργαστήριο Ρευματολογίας – Ανοσολογίας της Α΄ Προπαιδευτικής Παθολογικής Κλινικής της Ιατρικής Σχολής του Εθνικού και Καποδιστριακού Πανεπιστημίου Αθηνών κατά την χρονική περίοδο 2019 – 2024 υπό την επίβλεψη του Καθηγητή Παθολογίας – Ρευματολογίας κου Πέτρου Π. Σφηκάκη.

Ολοκληρώνοντας την διδακτορική μου διατριβή θα ήθελα να ευχαριστήσω μια σειρά ατόμων που μου έδειξαν τον δρόμο σε αυτό το συναρπαστικό ταξίδι στον κόσμο της έρευνας:

Αρχικά, τον Καθηγητή Παθολογίας – Ρευματολογίας Πέτρο Π. Σφηκάκη, τον επιβλέποντα της διατριβής αυτής, ο οποίος μετά το πέρας της Ιατρικής Σχολής, μου άνοιξε διάπλατα τις πόρτες του εργαστηρίου του, με βοήθησε να κάνω τα πρώτα μου βήματα στον χώρο της έρευνας και αποτελεί και θα αποτελεί τον μέντορα μου.

Τον Ερευνητή Α΄ Βασίλειο Σουλιώτη από το Εθνικό Ίδρυμα Ερευνών, ο οποίος όλα αυτά τα χρόνια μου μετέδωσε εκτός από τις ουσιώδεις γνώσεις του στον χώρο της Βιολογίας και την αγάπη του για την επιστήμη, ενώ βρισκόταν όλα αυτά τα χρόνια απρόσκοπτα στο πλευρό μου βοηθώντας με σε κάθε δυσκολία σε αυτό το ταξίδι.

Τον Καθηγητή Ιστολογίας Βασίλειο Γοργούλη, ο οποίος με τίμησε συμμετέχοντας σε αυτή την Τριμελή Συμβουλευτική Επιτροπή και μου επέτρεψε να βελτιωθώ περεταίρω επιστημονικά, διευρύνοντας τις δεξιότητες μου.

Την Καθηγήτρια Ρευματολογίας Μαρία Τεκτονίδου, η οποία σε όλη την διάρκεια της διατριβής μου επέδειξε ιδιαίτερο ενδιαφέρον για την πρόοδο των πειραμάτων και με εποικοδομητική κριτική της διεύρυνε τους ορίζοντες μου.

Τον Επίκουρο Καθηγητή Ρευματολογίας Γεώργιο Φραγκούλη, ο οποίος με βοήθησε σημαντικά στην στρατολόγηση των ασθενών και με τις επιστημονικές συζητήσεις μας συνέβαλε σημαντικά στο να δουλέψω ακόμα πιο σκληρά και συστηματικά.

Ευχαριστώ επίσης την καθηγήτρια Μαυραγάνη Κλειώ και τον καθηγητή Λυμπερόπουλο Ευάγγελο, οι οποίοι μου έκανα την τιμή και δέχτηκαν να συμμετάσχουν στην Επταμελή Εξεταστική Επιτροπή της διατριβής μου.

Ένα μεγάλο ευχαριστώ οφείλω να πω και στις γραμματείς της Α' Προπαιδευτικής Παθολογικής κλινικής και της Ρευματολογικής Μονάδας της Α΄ Προπαιδευτικής Παθολογικής Κλινικής, οι οποίες με το χαμόγελο και την προθυμία τους ήταν πάντα δίπλα μου σε κάθε ανάγκη που πρόκυπτε.

Επίσης, ένα ξεχωριστό ευχαριστώ θέλω να πω σε όλους τους ειδικευμένους και ειδικευόμενους Ρευματολόγους της Ρευματολογικής Μονάδας της Α΄ Προπαιδευτικής Παθολογικής Κλινικής, οι οποίοι με βοήθησαν έμπρακτα στην στρατολόγηση των ασθενών. Ιδιαιτέρως, θα ήθελα να ευχαριστήσω από καρδιάς τον μεταδιδάκτορα Νίκο Βλαχόγιαννη και την διδάκτορα Μαρία Παππά, οι οποίοι ήταν δίπλα μου στο εργαστήριο Ρευματολογίας από την πρώτη μέρα, μου στάθηκαν πραγματικά δίπλα μου, όποτε τους είχα ψυχολογικά ανάγκη και μπορώ να πω ότι κέρδισα δυο νέους φίλους.

Ευχαριστώ επίσης όλους τους ασθενείς, οι οποίοι δέχτηκαν να συμμετέχουν στην διδακτορική αυτή διατριβή και αποτελούσαν και θα αποτελούν πηγή έμπνευσης μου ως επιστήμονα και ως ιατρό.

Τέλος, το πιο μεγάλο ευχαριστώ οφείλω να πω στην οικογένεια μου. Η μητέρα μου Ελένη και η αδερφή μου Αθηνά, βρισκόταν πάντα στο πλευρό μου σε όλη αυτή την διαδρομή και με υποστήριζαν ουσιαστικά όποτε το χρειαζόμουν. Η σύντροφός μου, Άννα, συνέβαλε καθοριστικά, αφού με την ανοχή της και την αγάπη της, με ενδυνάμωνε στις πιο δύσκολες στροφές αυτού του ταξιδιού. Τέλος, ο εκλιπών πατέρας μου, Ανδρέας, στον οποίο και αφιερώνω αυτή την διατριβή, ήταν ο καθοριστικός παράγοντας, αφού παρατηρώντας το παράδειγμα του από την παιδική μου ηλικία, μου εμφύσησε την επιστημονική περιέργεια.

Αθήνα, Ιανουάριος 2024

Παναγιώτης Ιωάννης Ντούρος

Ιπποκράτειος Όρκος (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.

Ιπποκράτειος Όρκος (απόδοση στη νέα ελληνική γλώσσα)

Ορκίζομαι στο θεό Απόλλωνα τον ιατρό και στο θεό Ασκληπιό και στην Υγεία και στην Πανάκεια και επικαλούμενος τη μαρτυρία όλων των θεών ότι θα εκτελέσω κατά τη δύναμη και την κρίση μου τον όρκο αυτόν και τη συμφωνία αυτή.Να θεωρώ τον διδάσκαλό μου της ιατρικής τέχνης ίσο με τους γονείς μου και την κοινωνό του βίου μου. Και όταν χρειάζεται χρήματα να μοιράζομαι μαζί του τα δικά μου. Να θεωρώ την οικογένειά του αδέλφια μου και να τους διδάσκω αυτήν την τέχνη αν θέλουν να την μάθουν χωρίς δίδακτρα ή άλλη συμφωνία.
Να μεταδίδω τους κανόνες ηθικής, την προφορική διδασκαλία και όλες τις άλλες ιατρικές γνώσεις στους γιους μου, στους γιους του δασκάλου μου και στους εγγεγραμμένους μαθητές που πήραν τον ιατρικό όρκο, αλλά σε κανέναν άλλο.
Θα χρησιμοποιώ τη θεραπεία για να βοηθήσω τους ασθενείς κατά τη δύναμη και την κρίση μου, αλλά ποτέ για να βλάψω ή να αδικήσω. Ούτε θα δίνω θανατηφόρο φάρμακο σε κάποιον που θα μου το ζητήσει, ούτε θα του κάνω μια τέτοια υπόδειξη.
Παρομοίως, δεν θα εμπιστευτώ σε έγκυο μέσο που προκαλεί έκτρωση. Θα διατηρώ αγνή και άσπιλη και τη ζωή και την τέχνη μου. Δεν θα χρησιμοποιώ νυστέρι ούτε σε αυτούς που πάσχουν από λιθίαση, αλλά θα παραχωρώ την εργασία αυτή στους ειδικούς της τέχνης.
Σε όσα σπίτια πηγαίνω, θα μπαίνω για να βοηθήσω τους ασθενείς και θα απέχω από οποιαδήποτε εσκεμμένη βλάβη και φθορά, και ιδίως από γενετήσιες πράξεις με άνδρες και γυναίκες, ελεύθερους και δούλους. Και όσα τυχόν βλέπω ή ακούω κατά τη διάρκεια της θεραπείας ή και πέρα από τις επαγγελματικές μου ασχολίες στην καθημερινή μου ζωή, αυτά που δεν πρέπει να μαθευτούν παραέξω δεν θα τα κοινοποιώ, θεωρώντας τα θέματα αυτά μυστικά.
Αν τηρώ τον όρκο αυτό και δεν τον παραβώ, ας χαίρω πάντοτε υπολήψεως ανάμεσα στους ανθρώπους για τη ζωή και για την τέχνη μου. Αν όμως τον παραβώ και επιορκήσω, ας πάθω 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 will 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

Personal Information

Name: Panagiotis Ioannis Ntouros

Date of Birth: 25/07/1994

Place of Birth: Athens, Greece

Education

2012-2018	Medical Degree: Medical School, Democritus University of Thrace, Alexandroupolis Greece.

Clinical experience

9/2023 – now	Rural doctor in Vilia Regional Medical center and Elefsina Medical Center.
11/2022- 8/2023	Army duty – Medical doctor in the Nephrology Department of 251 Air Force General Hospital
8/2021 – 9/2022	Resident of Internal Medicine, General Hospital of Drama, Greece
12/2018- 6/2021	Research Associate, First Department of Propaedeutic Internal Medicine and Joint Rheumatology Program, National and Kapodistrian University of Athens Medical School

Presentations and Awards

2/2023	Oral presentation: Type-I interferon signature and DNA damage accumulation in peripheral blood of patients with psoriatic arthritis
12/2022	Poster: Oxidative Stress and defective DNA damage repair in patients with Antiphospholipid Syndrome

4/2022	Poster: Deregulated DNA Damage Response – Repair Network in Behcet's Disease
7-9/10/2020	Oral presentation: DNA damage accumulation and decreased DNA repair capacity in the elderly.

Publications in peer-reviewed journals

Original articles

1. Fragoulis GE*, **Ntouros PA***, Nezos A, Vlachogiannis NI, McInnes IB, Tektonidou MG, Skarlis C, Souliotis VL, Mavragani CP, Sfikakis PP. Type-I interferon pathway and DNA damage accumulation in peripheral blood of patients with psoriatic arthritis. Front Immunol. 2023 Dec 6;14:1274060. doi: 10.3389/fimmu.2023.1274060. PMID: 38124740; PMCID: PMC10731026. (*equal 1st author)

2. Pappa M*, **Ntouros PA***, Papanikolaou C, Sfikakis PP, Souliotis VL, Tektonidou MG. Augmented oxidative stress, accumulation of DNA damage and impaired DNA repair mechanisms in thrombotic primary antiphospholipid syndrome. Clin Immunol. 2023 Sep;254:109693. doi: 10.1016/j.clim.2023.109693. Epub 2023 Jul 16. PMID: 37454866. (*equal 1st author)

3. Vlachogiannis NI, **Ntouros PA**, Pappa M, Kravvariti E, Kostaki EG, Fragoulis GE, Papanikolaou C, Mavroeidi D, Bournia VK, Panopoulos S, Laskari K, Arida A, Gorgoulis VG, Tektonidou MG, Paraskevis D, Sfikakis PP, Souliotis VL. Chronological Age and DNA Damage Accumulation in Blood Mononuclear Cells: A Linear Association in Healthy Humans after 50 Years of Age. Int J Mol Sci. 2023 Apr 12;24(8):7148. doi: 10.3390/ijms24087148. PMID: 37108309; PMCID: PMC10138488.

4. Vlachogiannis NI*, **Ntouros PA***, Pappa M, Verrou KM, Arida A, Souliotis VL, Sfikakis PP. Deregulated DNA damage response network in Behcet's disease. Clin Immunol. 2023 Jan;246:109189. doi: 10.1016/j.clim.2022.109189. Epub 2022 Nov 16. PMID: 36400336. (*equal 1st author)

5. Kravvariti E, **Ntouros PA**, Vlachogiannis NI, Pappa M, Souliotis VL, Sfikakis PP. Geriatric Frailty Is Associated With Oxidative Stress, Accumulation, and Defective Repair of DNA Double-Strand Breaks Independently of Age and Comorbidities. J

Gerontol A Biol Sci Med Sci. 2023 Mar 30;78(4):603-610. doi: 10.1093/gerona/glac214. PMID: 36209410.

6. Sfikakis PP, Vlachogiannis NI, **Ntouros PA**, Mavrogeni S, Maris TG, Karantanas AH, Souliotis VL. Microvasculopathy-Related Hemorrhagic Tissue Deposition of Iron May Contribute to Fibrosis in Systemic Sclerosis: Hypothesis-Generating Insights from the Literature and Preliminary Findings. Life (Basel). 2022 Mar 16;12(3):430. doi: 10.3390/life12030430. PMID: 35330181; PMCID: PMC8955192.

7. **Ntouros PA**, Kravvariti E, Vlachogiannis NI, Pappa M, Trougakos IP, Terpos E, Tektonidou MG, Souliotis VL, Sfikakis PP. Oxidative stress and endogenous DNA damage in blood mononuclear cells may predict anti-SARS-CoV-2 antibody titers after vaccination in older adults. Biochim Biophys Acta Mol Basis Dis. 2022 Jun 1;1868(6):166393. doi: 10.1016/j.bbadis.2022.166393. Epub 2022 Mar 18. PMID: 35314351; PMCID: PMC8930778.

8. Vlachogiannis NI, Tual-Chalot S, Zormpas E, Bonini F, **Ntouros PA**, Pappa M, Bournia VK, Tektonidou MG, Souliotis VL, Mavragani CP, Stamatelopoulos K, Gatsiou A, Sfikakis PP, Stellos K. Adenosine-to-inosine RNA editing contributes to type I interferon responses in systemic sclerosis. J Autoimmun. 2021 Dec;125:102755. doi: 10.1016/j.jaut.2021.102755. Epub 2021 Nov 29. PMID: 34857436; PMCID: PMC8713031.

9. **Ntouros PA**, Vlachogiannis NI, Pappa M, Nezos A, Mavragani CP, Tektonidou MG, Souliotis VL, Sfikakis PP. Effective DNA damage response after acute but not chronic immune challenge: SARS-CoV-2 vaccine versus Systemic Lupus Erythematosus. Clin Immunol. 2021 Aug;229:108765. doi: 10.1016/j.clim.2021.108765. Epub 2021 Jun 2. PMID: 34089859; PMCID: PMC8171000.

10. Vlachogiannis NI, Pappa M, **Ntouros PA**, Nezos A, Mavragani CP, Souliotis VL, Sfikakis PP. Association Between DNA Damage Response, Fibrosis and Type I Interferon Signature in Systemic Sclerosis. Front Immunol. 2020 Oct 2;11:582401. doi: 10.3389/fimmu.2020.582401. PMID: 33123169; PMCID: PMC7566292.

Review articles

1. Souliotis VL, Vlachogiannis NI, Pappa M, Argyriou A, **Ntouros PA**, Sfikakis PP. DNA Damage Response and Oxidative Stress in Systemic Autoimmunity. Int J Mol Sci. 2019

Dec 20;21(1):55. doi: 10.3390/ijms21010055. PMID: 31861764; PMCID: PMC6982230.

Continuing education – biomedical congresses

23-24/6/2023	22nd Annual Hellenic Conference of Physical medicine and rehabilitation
8-11/12/2022	28th Congress of the Hellenic Society of Rheumatology, Athens, Greece.
11-13/10/2021	17th European Geriatric Medicine Society (EUGMS) Congress, Athens, Greece
12/2020	3rd Lupus School of the Athens Lupus Forum
7-9/10/2020	European Geriatric Medical Society E-congress, Covid-19: lessons and challenges for health care for older adults.
9-12/9/2019	Conference of the International Cell Senescence Association (ICSA), "The bright and dark side of cellular senescence", Biomedical Research Foundation of the Academy of Athens, September 2019.
6-9/12/2018	26th Congress of the Hellenic Society of Rheumatology, Athens, Greece
9/6/2018	Educational Seminar, Advisory skills and effective doctor to patient communication in patients with chronic illness.
10-12/2/2017	8o Cardiological Congress Eastern Macedonia and Thrace, "Treatment of Cardiological Diseases in 2017: The many-sided contribution of imaging"
26-27/9/2014	8o Annual Seminar in Electrolytes and acid-base balance, Hellenic Society of Nephrology.

Language skills

English	C2 – Michigan University
German	C1 – Goethe Institut

Scientific bodies

EMEUNET - EMerging EULAR NETwork

Athens Medical Association

Table of Contents

Στοιχεία Διδακτορική Διατριβής *3*

Ευχαριστίες *4*

Ιπποκράτειος Όρκος (αρχαία ελληνική γλώσσα) *6*

Ιπποκράτειος Όρκος (απόδοση στη νέα ελληνική γλώσσα) *7*

The Hippocratic oath *8*

Curriculum vitae *9*

List of abbreviations *16*

Summary *21*

Περίληψη (Summary in Greek) *23*

1. Introduction *25*

1.1 Comet assay as a biomarker of DNA damage **28**

1.2 Abasic site quantification as a biomarker of DNA damage **29**

1.3 Oxidative stress contributes to DNA damage formation **30**

1.3.1 Glutathione as a biomarker of oxidative stress 32

1.4 The effect of age on oxidative stress and DNA damage response **33**

1.5 The effect of DNA damage response and oxidative stress in the development of systemic autoimmune diseases **34**

1.5.1 Systemic Lupus Erythematosus 36

1.5.2. Rheumatoid Arthritis 40

1.5.3 Systemic Sclerosis 43

1.5.4 Adamantiades – Behcet's Disease 47

1.5.5 Antiphospholipid Syndrome 49

2. Rationale and Purpose of the study *52*

3. Study population and methodology *54*

3.1 Study population **54**

3.2 Isolation of peripheral blood mononuclear cells **55**

3.2 DNA damage measurement by alkaline comet assay **57**

3.3 Oxidative stress quantification by measurement of glutathione (GSH) and oxidized glutathione (GSSG)......59

3.4 Abasic site quantification......61

3.5 Study of double-strand DNA break repair mechanism (DSB-R)......62

3.6 Statistical analysis......63

4. Results......64

4.1 Study population demographics and clinical characteristics......64

4.2 Increased levels of oxidative stress in patients with systemic autoimmune diseases. 68

4.3 Increased abasic site formation in patients with systemic autoimmune diseases..70

4.3 The interplay of the intracellular oxidative stress and DNA damage formation..72

4.4 Lack of association between chronological age and oxidative stress in patients with systemic autoimmune diseases......74

4.5 DNA damage accumulation in patients with systemic autoimmune diseases......76

4.6 DNA damage accumulation significantly correlates with intracellular oxidative stress in patients with systemic autoimmune diseases......78

4.7 The interplay of intracellular oxidative stress and DNA damage repair capacity 80

4.8 Lack of association between DNA damage repair capacity and aging in patients with systemic autoimmune diseases......82

4.9 Lack of association between endogenous DNA damage levels and aging in patients with systemic autoimmune diseases......84

5. Discussion......86

6. Conclusion......89

Bibliography......90

List of abbreviations

Abbreviation	Explanation
UV-c	Ultra violet radiation c
DDR	DNA damage response
DDR/R	DNA damage response and repair
FA	Fanconi anemia
RA	Rheumatoid Arthritis
SLE	Systemic Lupus Erythematosus
SSc	Systemic Sclerosis
ROS	Reactive Oxygen Species
RNS	Reactive Nitrogen Species
RSS	Reactive Sulfur Species
RCS	Reactive Chlorine Species
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NADP	nicotinamide adenine dinucleotide phosphate
NOX	NADPH oxidase
mTOR	mechanistic Target of Rapamycin
Nrf2	nuclear factor erythroid 2–related factor 2
AP-1	Activator protein 1
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
IL-2	Interleukin-2
IL-10	Interleukin-10
TNF-a	Tumor Necrosis Factor-a
BER	Base Excision Repair
AP site	apurinic/apyrimidinic site
O^-	superoxide

Abbreviation	***Explanation***
•OH	hydroxyl radical
H2O2	hydrogen peroxide 2
SSB	Single-strand DNA break
DSB	Double-strand DNA break
8-oxoGua	8-oxo-7,8-dihydroguanine
FAPγG	2,6-diamino-4-hydroxy-5-formamido-pyrimidine
thymine glycol	5,6-dihydroxy-5,6-dihydrothymine
cytosine glycol	,6-dihydroxy5,6-dihydrocytosine
GSH	Glutathione
GSSG-	Glutathione disulfide
Met	Methionine
Cys	Cystein
GR	GSH reductase
ALS	amyotropic lateral sclerosis
HPLC	high-performance liquid chromatography
LC-MS/MS	liquid chromatography -tandem mass spectrometry
p53	Tumor protein P53
CDK-I	cyclin-dependent kinase inhibitor 1
IκBs	Inhibitors of kB
IL-8	Interleukin-8
MAPK	mitogen-activated protein kinase
μRNA	micro RNA
RANTES	Regulated on Activation, Normal T Cell Expressed and Secreted
MCP-1	Monocyte Chemoattractant Protein-1
CRP	C-reactive protein
GWAs	Genome-wide association studies
IRF-5	Interferon Regulatory Factor 5

Abbreviation	Explanation
MHC	major histocompatibility complex
IRF-7	Interferon Regulatory Factor 7
EBV	Epstein–Barr virus
IFN	interferon
TLR	Toll-like receptor
RIG-1	retinoic acid-inducible gene I
MDA-5	melanoma differentiation-associated protein 5
cGAS	Cyclic GMP-AMP synthase
ΔΨm,	mitochondrial transmembrane potential
ROI	reactive oxygen intermediates
PBL	peripheral blood lymphocytes
DSB-R	double-strand break repair
RF	rheumatoid factor
DSB-R	double-strand break repair
RF	rheumatoid factor
anti-CCP	anti-cyclic citrullinated peptides
anti-Ro/ anti-SSA	Anti-Sjögren's-syndrome-related antigen A
anti-La / anti-SSB	anti-Sjögren's-syndrome related antigen B
ATM	ataxia telangiectasia mutated serine/threonine kinase
lc-SSc	limited cutaneous systemic sclerosis
dc-SSc	diffuse cutaneous systemic sclerosis
EULAR	European League Against Rheumatism
ACR	American College of Rheumatology
DAMPs	damage-associated molecular patterns
TGF-β	transforming growth factor - β
ECM	extracellular matrix
MDA	malondialdehyde
TAC	total antioxidant capacity

Abbreviation	Explanation
ABD	Adamantiades – Behcet's Disease
PDGF	Platelet-derived growth factor
PRR	Pathogen recognition receptors
HSV	Herpes simplex virus
APS	Antiphospholipid syndrome
aPL	antiphospholipid antibodies
PAMPs	pathogen associated molecular patterns
anti-β2GPI	anti-beta-2 glycoprotein I antibodies
pAPS	primary APS
sAPS	secondary APS
cAPS	catastrophic APS
thrAPS	Thrombotic APS
STAT4	signal transducer and activator of transcription-4
VDRL	Venereal Disease Research Laboratory
VZV	Varicella Zoster Virus
CMV	Cytomegalovirus
ICAM-I	intercellular cell adhesion molecule-1
VCAM-I	vascular cell adhesion molecule-1
PON-1	paraoxonase-1
NO	nitric oxide
ONOO−	Peroxynitrite
HC	Healthy controls
PBMC	Peripheral blood mononuclear cell
EDTA	Ethylenediaminetetraacetic acid
PBS	Phosphate Buffer Saline
FBS−	Fetal Bovine Serum
DMSO	Dimethylsulfoxide
NaOH	Sodium hydroxide

Abbreviation	Explanation
ARP	Aldehyde Reactive Probe
OTM	Olive Tail Moment
dsDNA	Double-strand DNA
AOPP	advanced oxidation protein products
ssDNA	Single-strand DNA
RPMI	Roswell Park Memorial Institute (RPMI) medium
HCl	hydrochloric acid
FITC	fluorescein isothiocyanate
Ser	serine
TRITC	tetramethylrhodamine
SD	Standard deviation
SOD	superoxide dismutase
GPx	glutathione peroxidase
GR	glutathione reductase
Prxs	peroxiredoxins
CAT	catalase
Mn	Manganese
Zn	Zinc
Cu	Copper
Se	Selenium

Summary

Systemic autoimmune diseases comprise an heterogenous group and are characterized by aberrant chronic immune activation against self-antigens, production of autoantibodies and tissue injury. Although their pathophysiology remains unclear, several lines of evidence suggest that the imbalance between the oxidant and antioxidant cellular systems after exposure to deleterious stimuli, termed oxidative stress, is shown to be involved. Reactive oxygen and nitrogen species are key mediators of oxidative stress and may damage cellular DNA. To protect the genome, cells have developed several DNA repair mechanisms, composing the DNA damage response and repair network (DDR/R). On the other hand, chronological age, associated with a decline of the immune system functionality, leading to increased susceptibility to cancer and autoimmunity. Herein, we aimed to examine whether systemic autoimmunity is associated with aberrations in oxidative stress formation and DNA damage accumulation, as well as whether these aberrations may be attributed to the person's chronological age.

We studied 78 consecutive patients with systemic autoimmune diseases, including Rheumatoid Arthritis (RA) (n=9), Systemic Lupus Erythematosus (SLE) (n=14), Systemic Sclerosis (SSc) (n=9), Adamantiades – Behcet's disease (ABD) (n=6) and Antiphospholipid Syndrome (APS) (n=40). A total of 212 apparently healthy individuals (HC) were studied in parallel. Peripheral blood mononuclear cells (PBMCs) were isolated using standard methods. Oxidative stress formation was assessed by quantifying intracellular glutathione oxidation with chemiluminescent methods , DNA damage levels were measured by alkaline single-cell gel electrophoresis (single- and double- strand DNA breaks) and abasic (apurinic/apyrimidinic) sites using a chemiluminescent assays, while DNA damage repair efficacy was assessed by immunofluorescence antigen staining and confocal laser microscopy (double-strand break repair / DSB-R). Results from each patient subgroup were compared with corresponding results derived from an HC subgroup, matched 1:3 for age and sex.

Firstly, increased oxidative stress levels were observed in RA, SLE, SSc, ABD and APS patients compared to HC (glutathione oxidation: RA: 46.2 ± 8,56 / HC: 70±10.7 [$p<0.001$], SLE: 49.86 ± 12.6 / HC: 70 ±8.6 [$p<0.001$], SSc: 52 ± 15.68 / HC: 67.29 ± 10.1, [$p<0.01$], ABD: 30.83 ± 8.33/ HC: 74.2 ± 8.54 [$p<0.001$] , APS: 44.86 ± 11.54 / HC: 69±11.33 [$p<0.001$])

Secondly, increased abasic site formation was observed in RA, SLE, SSc, ABD and APS patients compared to HC (abasic site formation: RA: 16.3 ± 3.2/ HC: 7.8±2.4 [p<0.001], SLE: 14.8±4 / HC: 7±2.4 [p<0.001], SSc: 12.56 ± 3.2 / HC: 7 ±2.4 [p<0.01], ABD: 23 ± 8.12/ HC: 5.7 ± 1.4 [p<0.001], APS: 16.6 ± 6.7/ HC: 7.3 ± 3.04 [p<0.001]). Abasic site formation strongly associated with intracellular oxidative stress levels in HCs and patients with systemic autoimmune diseases (HC: r=-0.555, p<0.001, Patients: r=-0.418, p<0.001). Thirdly, endogenous double-strand and single-strand DNA breaks were highly elevated in the PBMCs of patients with systemic autoimmune diseases, compared to HC (Olive Tail Moment: RA: 15.7±8.5 / HC: 6.2±1.7 [p<0.001], SLE: 9.7± 5.4 / HC: 4.7 ± 1.5 [p<0.001], SSc: 11.8 ± 8 / HC: 4.8± 1.8 [p<0.001], ABD: 9.4± 5.7 / HC: 4± 1.3 [p<0.01], APS: 14.5 ± 7.4 / HC: 5.3 ± 2 [p<0.001]). Collectively within patients, these elevated DNA damage levels strongly correlated with both the oxidative stress (r=-0.388 , p<0.001) and abasic site levels (r=0.455, p<0.001) underlining a possible mechanistic link. Notably, a lack of correlation was observed between DNA damage repair capacity and oxidative stress levels in patients. DNA damage repair capacity correlated strongly with oxidative stress levels (r=-0.500, p<0.001) in healthy individuals, indirectly confirming the deficient DDR capacity in patients with systemic autoimmunity. Finally, chronological age was found to strongly associate with oxidative stress (r=0.636, p<0.001), DNA damage levels (r=0.641, p<0.001) and DDR capacity (r=0.781 , p<0.001) in HCs, but not in patients with systemic autoimmune diseases.

In conclusion, we show that patients with systemic autoimmune diseases display increased oxidative stress, leading to increased DNA damage formation, and decreased DNA damage repair capacity. Such aberrations are not systemic autoimmune disease specific and are not influenced by the chronological age of the patient, as happens in the absence of autoimmunity.

Περίληψη (Summary in Greek)

Τα συστηματικά αυτοάνοσα νοσήματα συνιστούν μια ετερογενή ομάδα παθήσεων, οι οποίες χαρακτηρίζονται από χρόνια παρεκκλίνουσα ανοσολογική ενεργοποίηση έναντι αυτό-αντιγόνων, παραγωγή αυτοαντισωμάτων και ιστική καταστροφή. Αν και η παθοφυσιολογία της αυτοανοσίας παραμένει σκοτεινή, η ανισορροπία των οξειδωτικών και αντιοξειδωτικών κυτταρικών μηχανισμών μετά την έκθεση του κυττάρου σε επιβλαβή ερεθίσματα, που καλείται οξειδωτικό στρες, φαίνεται να συνεισφέρει στην κλινική εκδήλωση της αυτοανοσίας. Οι ελεύθερες ρίζες οξυγόνου και αζώτου, οι κύριοι οξειδωτικοί μεσολαβητές, μπορούν να μεταβάλουν την σύσταση του γονιδιώματος του κυττάρου, οδηγώντας σε βλάβες του DNA. Για την προστασία του γονιδιώματος, έχει αναπτυχθεί μια πληθώρα επιδιορθωτικών μηχανισμών του DNA που καλείται δίκτυο απόκρισης και επιδιόρθωσης βλαβών του DNA. Παράλληλα, η χρονολογική ηλικία του ατόμου έχει συσχετιστεί με την γήρανση του ανοσολογικού συστήματος, οδηγώντας σε αυξημένη πιθανότητα για καρκίνο και αυτοανοσία. Στην παρούσα μελέτη υποθέσαμε ότι τα συστηματικά αυτοάνοσα νοσήματα συσχετίζονται με ανωμαλίες του κυτταρικού οξειδωτικού στρες και αυξημένη συσσώρευση βλαβών του DNA και επιπλέον ελέγξαμε εάν οι παρεκκλίσεις συνδέονται με την ηλικία των ασθενών.

Στην μελέτη αυτή αναλύσαμε 78 διαδοχικοί ασθενείς με συστηματικά αυτοάνοσα νοσήματα, συμπεριλαμβανομένων ασθενών με Ρευματοείδη Αρθρίτιδα (ΡΑ) (N=9), με Συστηματικό Ερυθηματώδη Λύκο (ΣΕΛ) (N=14), Συστηματική Σκλήρυνση (ΣΣ) (N=9), με Νόσο Αδαμαντιάδη – Behcet's (ΝΒ) (N=6) και με Αντιφωσφολιπιδικό σύνδρομο (ΑΦΣ) (N=40). Παράλληλα, ένα σύνολο 212 φαινομενικά υγειών ατόμων (ΥΜ) αναλύθηκαν. Περιφερικά πολυμορφοπύρηνα κύτταρα απομονώθηκαν με τις συνήθεις μεθόδους. Το οξειδωτικό στρες ποσοτικοποιήθηκε μετρώντας την οξείδωση της ενδοκυττάριας γλουταθειόνης με μεθόδους χημειοφωταύγειας, τα επίπεδα βλαβών του DNA ποσοτικοποιήθηκαν μέσω της αλκαλικής ηλεκτροφόρησης μοναδιαίων κυττάρων (δίκλωνα και μονόκλωνα θραύσματα DNA) και οι αβασικές (απουρινικές / απυριμιδινικές) θέσεις μέσω μεθόδων χημειοφωταύγειας, ενώ η ικανότητα επιδιόρθωσης του DNA εκτιμήθηκε με μεθόδους ανοσοφθορισμού και συνεστιακής μικροσκοπίας. Τα αποτελέσματα από κάθε υποομάδα ασθενών συγκρίθηκαν με αντίστοιχη υποομάδα υγειών ατόμων, αφού είχε προηγηθεί 1:3 αντιστοιχία ηλικίας και φύλου.

Πρώτον, αυξημένα επίπεδα οξειδωτικού στρες παρατηρήθηκαν σε ασθενείς με ΡΑ, ΣΕΛ, ΣΣ, ΝΒ και ΑΦΣ σε σύγκριση με ΥΜ (οξείδωση γλουταθειόνης: ΡΑ: 46.2 ± 8,56 / ΥΜ: 70±10.7

[p<0.001], ΣΕΛ: 49.86 ± 12.6 / ΥΜ: 70 ±8.6 [p<0.001], ΣΣ: 52 ± 15.68 / ΥΜ: 67.29 ± 10.1, [p<0.01] ΝΒ: 30.83 ± 8.33/ ΥΜ: 74.2 ± 8.54 [p<0.001] , ΑΦΣ: 44.86 ± 11.54 / ΥΜ: 69±11.33 [p<0.001]). Δεύτερον, αυξημένα επίπεδα σχηματισμού αβασικών θέσεων παρατηρήθηκαν σε ασθενείς με ΡΑ, ΣΕΛ, ΣΣ, ΝΒ και ΑΦΣ σε σύγκριση με ΥΜ (σχηματισμός αβασικών θέσεων: ΡΑ: 16.3 ± 3.2/ ΥΜ: 7.8±2.4 [p<0.001], ΣΕΛ: 14.8±4 / ΥΜ: 7±2.4 [p<0.001], ΣΣ: 12.56 ± 3.2 / ΥΜ: 7 ±2.4 [p<0.01], ΝΒ: 23 ± 8.12/ ΥΜ: 5.7 ± 1.4 [p<0.001], ΑΦΣ: 16.6 ± 6.7/ ΥΜ: 7.3 ± 3.04 [p<0.001]). Τρίτον, τα ενδογενή επίπεδα δίκλωνων και μονόκλωνων θραυσμάτων του DNA στους ασθενείς με τα εξεταζόμενα αυτοάνοσα νοσήματα ήταν σημαντικά αυξημένα σε σύγκριση με τους ΥΜ (Olive Tail Moment: ΡΑ: 15.7±8.5 / ΥΜ: 6.2±1.7 [p<0.001], ΣΕΛ: 9.7± 5.4 / ΥΜ: 4.7 ± 1.5 [p<0.001], ΣΣ: 11.8 ± 8 / ΥΜ: 4.8± 1.8 [p<0.001], ΝΒ: 9.4± 5.7 / ΥΜ: 4± 1.3 [p<0.01], ΑΦΣ: 14.5 ± 7.4 / ΥΜ: 5.3 ± 2 [p<0.001]). Συνολικά στους ασθενείς, τα αυξημένα επίπεδα των βλαβών του DNA ισχυρά συσχετίζονται τόσο με το οξειδωτικό στρες (r=-0.388 , p<0.001) όσο και με τα επίπεδα των αβασικών θέσεων (r=0.455, p<0.001), υποδηλώνοντας έναν πιθανό παθογενετικό μηχανισμό. Ιδιαιτέρα, η κυτταρική ικανότητα επιδιόρθωσης του DNA δεν φαίνεται να συσχετίζεται με τα επίπεδα του οξειδωτικού στρες στου ασθενείς, σε αντίθεση με τους υγιείς μάρτυρες, εμμέσως επαληθεύοντας την μειωμένη ικανότητα επιδιόρθωσης στους ασθενείς με συστηματική αυτοανοσία. Τέλος, η ηλικία του ατόμου φαίνεται να συσχετίζεται σημαντικά τόσο με τα επίπεδα του οξειδωτικού στρες (r=0.636, p<0.001), τα επίπεδα βλαβών του DNA (r=0.641, p<0.001) και την κυτταρική ικανότητα επιδιόρθωσης του DNA (r=0.781 , p<0.001) στους υγιείς μάρτυρες αλλά όχι στους ασθενείς με συστηματικά αυτοάνοσα νοσήματα.

Συνοψίζοντας, στην μελέτη αυτή δείχνουμε ότι οι ασθενείς με συστηματικά αυτοάνοσα νοσήματα εμφανίζουν αυξημένο οξειδωτικό στρες, αυξημένο σχηματισμό βλαβών του DNA και μειωμένη ικανότητα επιδιόρθωσης του DNA. Αυτές οι διαταραχές φαίνεται να είναι μη-ειδικές για τα εξεταζόμενα συστηματικά αυτοάνοσα νοσήματα, αλλά και να μην επηρεάζονται από την ηλικία του ασθενούς, όπως συμβαίνει σε άτομα χωρίς αυτοανοσία.

TITLE: Oxidative stress and the DNA damage Response/Repair network in Systemic Autoimmune Diseases

THEORETICAL BACKGROUND

(Γενικό μέρος)

1. Introduction

The human body consists of 10^{13} cells, containing $3x10^9$ base pairs and coding more than 30.000 genes. However, the cells of the human body receive approximately 70.000 DNA lesions every day. These DNA lesions can obstruct the genome replication and transcription, if not successfully repaired, and may lead to mutations or wide-scale genomic instability. It was known, even before the elucidation of the DNA structure by Watson and Crick in 1953, that these DNA lesions can arise from exogenous factors, such as ionizing radiation (X-rays, Ultraviolet radiation) and/or various chemicals (mutagenic and chemotherapeutic agents). After the discovery of the stable double-helix structure of the DNA, it was recognized that DNA is also subject to constant DNA damage from endogenous sources, produced even during normal metabolic operations (1-3).

Even though the ozone layer absorbs the most harmful part of the solar ultraviolet spectrum (Ultra-Violet radiation c / UV-c), the residual solar UV radiation spectrum constitutes the most pervasive DNA-damaging agent (3,4). Exposure to natural (uranium decay, radon gas) or man-made (used during radiotherapies) radioisotopes can generate DNA damage via ionizing radiation. In addition, chemical sources can induce DNA damage. Chemical agents used in chemotherapy (alkylating and crosslinking agents or topoisomerase inhibitors) can cause severe DNA damage. However, today, the most prevalent chemical agents causing self-inflicting DNA damage are directly connected with the tobacco smoke, related with many types of cancer most notably of the lungs, oral cavity and adjacent tissues (5,6).

Some DNA damage lesions can arise, apart from the aforementioned exogenous agents, via physiological processes. During DNA replication DNA mismatches can occur occasionally. A primary example is the abortive function of topoisomerase I, causing DNA-strand breaks. Most

importantly, normal cellular metabolism is a well-defined source of endogenous reactive oxygen species and is accountable for the oxidative DNA damage (7,8).

Upon DNA damage cells respond by initiating a robust DNA damage response and repair network, allowing sufficient time to repair. This DNA damage response and repair network (DDR/R) is a hierarchically structured network that consists of sensors, mediators, transducers and effectors, which recognize any DNA lesion in a substrate-specific manner and assign the specific DNA repair process (7,9). In case of unrepaired DNA lesions and depending on the severity of the damage, cell either transfers the mutated genetic material to its offspring or undergoes either programmed cell death (apoptosis) or senescence (6,10).

The majority of the studies researching defects on the DDR/R network are focused on malignancies and its implications on the state-of-the-art cancer therapies on inherited diseases, where mutations on key-genes related to DDR are observed, such as Fanconi anemia (FA) or Ataxia-Telangiectasia, on neurodegenerative diseases, such as Alzheimer's, Huntington's or Parkinson's disease, which are related with DNA damage accumulation in neurons (11-16). However, recent studies have reported that patients with autoimmune diseases exhibit deficiencies in the DDR/R network. These aberrations are implicated in the perpetuation and pathogenesis of many autoimmune diseases, such as Rheumatoid Arthritis (RA), Systemic Lupus Erythematosus (SLE) or Systemic Sclerosis (SSc) (17-20).

Oxidative stress occurs when the balance between the production and elimination of reactive oxygen, nitrogen, sulfur and chlorine species (ROS, RNS, RSS, RCS accordingly) is vanished. ROS and RNS, the two key representors of oxidative stress, can be generated under normal circumstances via cellular metabolism and respiration with the use of mitochondrial electron transport chain, NADPH oxidases (NOX), nitric oxide synthases, and nitrite reductases.

However, in autoimmune diseases oxidative species can be overproduced due to systemic inflammation (21,22). ROS are produced by host phagocytes and exert antimicrobial actions against a broad range of pathogens during an inflammatory response. Thus, generation of oxidative species can be beneficial for the host defense against microbial infection, but inappropriate generation of ROS, as in autoimmune diseases, can affect and damage host tissue.

Oxidative stress may lead to upregulation of intracellular inflammatory pathways, such as rapamycin (mTOR) activity, affecting redox-sensitive transcription factors (Nrf2, AP-1 and NFκB) leading to cytokine production (IL-2, IL-10, and TNF-a) and further perpetuating autoimmune inflammation (23-25).

The cell can eliminate high levels of oxidative species with the use of scavenging systems. There have been developed several mechanisms, providing elimination of these free radicals and their derivatives. Some of these systems may contain enzymatic antioxidant molecules, such as superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR), peroxiredoxins (Prxs) and catalase (CAT), interrupting the oxidation reaction and repairing the oxidation products, or non-enzymatic endogenous substances, such as the proteins ceruloplasmin, ferritin, transferrin and albumin, and dietary antioxidants such as vitamin C and E, carotenoids, minerals (Mn, Zn, Cu, Se) and polyphenols (flavonoids, phenolic acids, stilbenes, lignans), with their main goal being to prevent the formation of the free radicals (200-202).

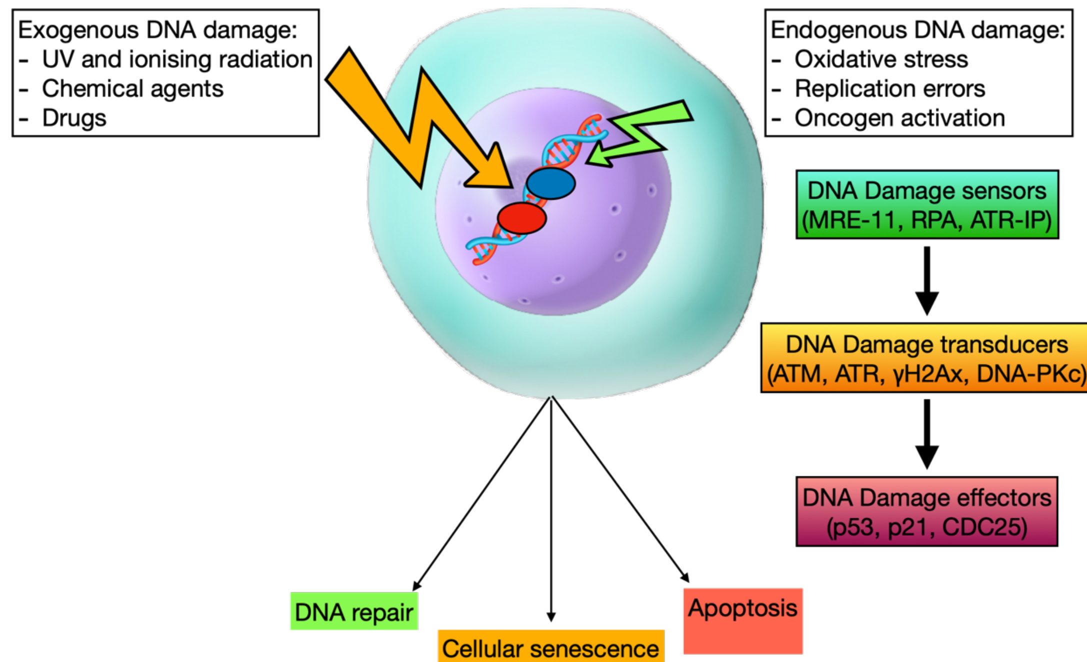


Figure 1 : *Graphic illustration of DNA Damage Response and Repair network (DDR/R).*

1.1 Comet assay as a biomarker of DNA damage

It is generally accepted that several insults, endogenous or exogenous, may damage the genomic DNA. This DNA damage can influence the integrity of the DNA molecule and lead to DNA base damage, DNA sugar damage , single-strand DNA breaks or double-strand DNA breaks, the latter being the most cytotoxic. Hence, the measurement of double-strand DNA breaks is of outmost importance, since these lethal DNA nicks can allow the prediction of cell death. There have been developed a series of methods to quantify double-strand DNA breaks, such as neutral elution, pulse field electrophoresis (2-D gel electrophoresis) and the comet assay (single-cell gel electrophoresis).

Comet assay is a popular method to quantify DNA strand breaks, due to its low cost, high reliability and versatility. The method was first mentioned in 1987 in a research paper by Singh *et. al*, where the researchers measured the length of DNA migration in photomicrographs, a method named “single-cell microgel electrophoresis technique”. The term comet assay was introduced in 1990 by Olive *et. al* , describing the shape of DNA migration in agarose gels.

The final method used today, named alkaline comet assay, is based on the ability of negatively-charged fragments of DNA, created by the DNA strand breaks, to be attracted in response to an electric field through an agarose gel. This alkaline version of comet assay mainly detects DNA lesions that are converted to DNA strand breaks by the alkaline conditions of the assay. These DNA lesions, named alkali labile sites, are native DNA motifs highly sensitive to incubation in alkali solutions which produce DNA strand breaks. These motifs mainly include abasic site areas. The DNA molecule under the alkaline conditions of the method denatures and is subjected to electrophoresis, allowing both the DNA loops and fragments to migrate towards the anode. Therefore, the fraction of the DNA in the DNA migration tail is proportional to the number of the alkali-labile sites and the endogenous DNA strand breaks.

1.2 Abasic site quantification as a biomarker of DNA damage

DNA sustains a wide variety of DNA damage, upon exposure to cellular oxidative stress. The DNA lesions associated with oxidative stress, and mainly with reactive Oxygen and Nitrogen species (ROS and RNS respectively), are oxidized purines and pyrimidines, abasic sites and the severely cytotoxic double- and single- strand DNA breaks. DNA abasic site formation results from nucleotide depyrimidation or depurination. Under physiological conditions, cellular DNA depyrimidation and depurination is a rare event, estimating a rate of 3 x 10^{-11} abasic sites x s^{-1}. This base loss phenomenon is promoted by oxidation of the DNA molecule or can be an intermediate product in DNA repair mechanisms, such as the Base Excision Repair (BER) mechanism.

Abasic sites can manifest in a variety of forms, such as 2′-deoxyribonolactone and C4-AP site, directly resulting from nucleobase hydrolysis(26). 2′-deoxyribonolactone (L) is produced by hydrogen abstraction from hydroxyl radicals at the C1 of the 2-deoxyribose, while C4-AP is produced by hydrogen abstraction at the C4 of the 2-deoxyribose moiety of DNA(27,28).

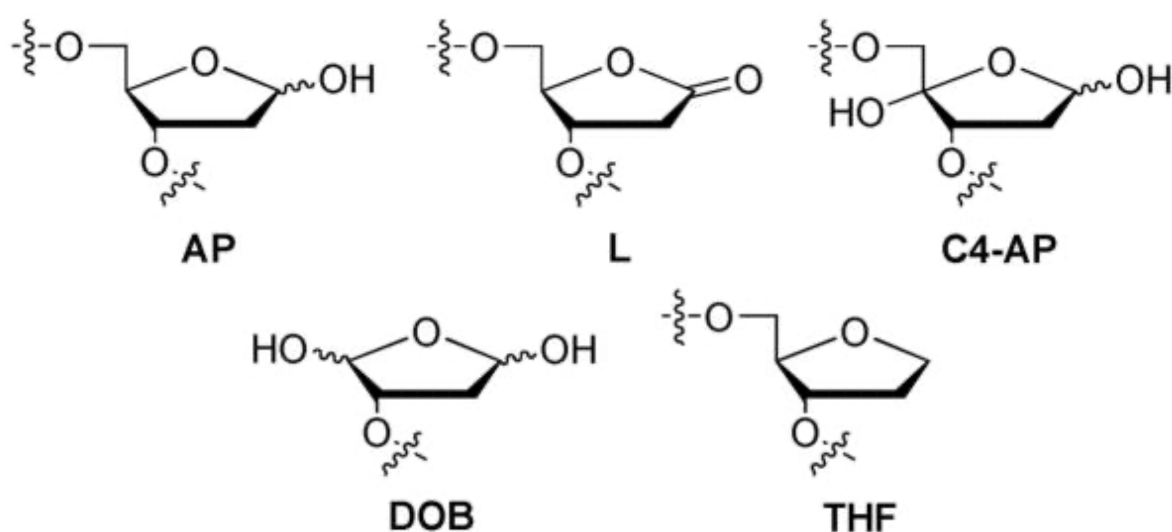


Figure 2*: Depiction of the variety of structural forms of abasic DNA lesions.*

1.3 Oxidative stress contributes to DNA damage formation

Cellular oxidative stress is a major cause of cellular and tissue injury, mainly caused by free radicals in the form of ROS and RNS. These radicals are oxygen- or nitrogen- containing, highly chemically reactive molecules that are the typical byproducts of the electron transport chain during cellular respiration in aerobic organisms and are additionally derived from catabolic oxidases, anabolic processes and peroxisomal metabolism (29,30).

The major types of ROS include superoxide ($O^{\bullet -}$), hydrogen peroxide 2 (H2O2), and hydroxyl radical (•OH). Mitochondria are the most important source of intracellular ROS. $O^{\bullet -}$ are generated when an oxygen (O) molecule gains one electron via electron leakage from the respiratory chain. ROS, when kept at low levels, are essential signaling molecules involved in the induction of many cellular or physiological activities such as messengers in redox signalling reactions and effectors in immune responses to invading pathogens and in cellular apoptosis (29,31,32).

The term oxidative stress, firstly mentioned by Helmut Sies in 1985, describes an imbalance between the production of oxidative species and the antioxidant cellular capacity, and may result in deleterious damage in many intracellular macromolecules (33,34). This redox imbalance due to ROS accumulation can be noticed in many pathological conditions such as during inflammation and aging (34-36). Increased ROS can be produced by activated inflammatory cells, such as macrophages, neutrophils and eosinophils, promoting endothelial dysfunction (oxidation of crucial cellular signaling proteins such as tyrosine phosphatases), further propagating the inflammation, as well as during the metabolism of xenobiotics by cytochrome-P450 oxidoreductases (37,204).

Oxidative stress and ROS accumulation can cause a great variety of oxidative DNA lesions. The main DNA lesions associated with ROS are oxidized purines and pyrimidines, abasic sites, SSBs and DSBs. The endogenous DNA base modifications may be produced by hydroxyl radicals, adding to guanine and adenine at positions 4,5, or 8 in the purine ring, generating 8-oxo-7,8-dihydroguanine (8-oxoGua) and 2,6-diamino-4-hydroxy-5-formamido-pyrimidine (FAPγG) (38). The most frequent oxidative lesion is 8-oxo-7,8-dihydroguanine, also called 8-hydroxyguanine, , due to its low oxidation reduction potential, is mainly mispaired with adenine (39,203). In addition, ROS can mediate pyrimidine oxidation, which occurs at

positions 5 or 6 in the pyrimidine ring, producing several radical species, such as 5,6-dihydroxy-5,6-dihydrothymine (thymine glycol) and 5,6-dihydroxy5,6-dihydrocytosine (cytosine glycol) (40). Two other pyrimidine lesions are the 5-hydroxymethyl uracil and the 5-formyluracil, when detected in humans can be the result of the oxidation of the methyl group of thymine. The hydroxyl radicals interacting with the DNA bases can mediate the production of single-strand DNA breaks (SSBs) and double-strand DNA breaks (DSBs). Mechanistically, hydrogen abstraction from the 2-deoxyribose may lead to the formation of carbon-based radicals and be converted to peroxyl radicals under the presence of oxygen. The peroxyl radicals also abstract hydrogen from sugar moieties, finally leading to DNA strand breaks (41).

However, when cells were exposed to cellular oxidative stress, they can initiate a variety of both enzymatic and non-enzymatic anti-oxidant defensive mechanisms. The enzymatic antioxidants include superoxide dismutase, glutathione peroxidase, glutathione reductase, glutathione-S-transferase, and catalase, whereas non-enzymatic antioxidants contain ascorbic acid (vitamin C), α-tocopherol (vitamin E), total thiol, glutathione, carotenoids, and flavonoids (42).

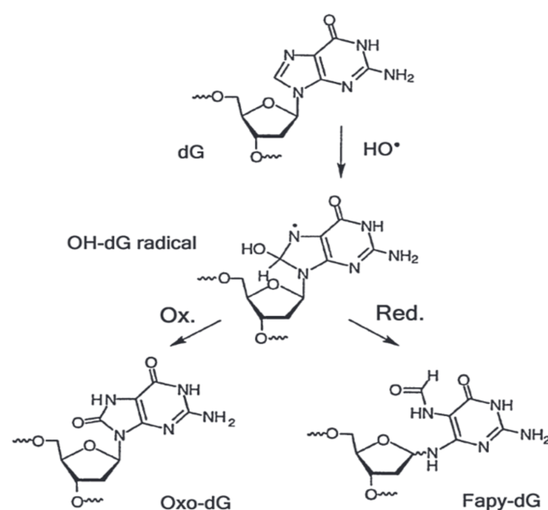


Figure 3 : *Graphic illustration of Guanine modification by hydroxyl radicals and production of 8-oxo-7,8-dihydroguanine (8-oxoGua) and 2,6-diamino-4-hydroxy-5-formamido-pyrimidine (Fapγ-dGua).*

1.3.1 Glutathione as a biomarker of oxidative stress

Glutathione (GSH) is the most-abundant thiol-containing residue in every cellular type and plays a central role in maintaining the physiological intracellular redox status *in vivo*. The highest concentrations of GSH are observed in the liver and kidney, mainly produced by the transsulfuration pathway (Met → homo-Cys → Cys), while lower levels are present in the brain cells, where the production of GSH is independent of the beforementioned pathway (43). GSH is mainly (85-95%) localized in the cytoplasm, with the remainder distributed in the peroxisomes, the nuclear matrix, endoplasmic reticulum, and the mitochondria (44,45).

Under cellular oxidative stress, ROS and RNS interact with the GSH, producing molecules of GSH disulfide (GSSG). This oxidized form can be reverted back to its reduced state via reaction with GSH reductase, in order to be maintained a redox homeostasis. Under normal circumstances, the intracellular GSH/GSSG ratio exceeds 100, declining to below 10 under highly oxidative stress conditions (46). Of note, many genetic mitochondrial disorders are associated both with dysregulations of the mitochondrial electron transport chain and low intracellular GSH levels, such as Friedreich ataxia, Alzheimer disease, Parkinson disease, amyotrophic lateral sclerosis (ALS), and Rett syndrome (47).

However, GSH quantification can oppose many challenges, because of the instability of GSH and the variety of methods between the laboratories, including high-performance liquid chromatography (HPLC), gas chromatography with mass spectrometry, capillary electrophoresis with ultraviolet absorbance or colorimetric detection, and liquid chromatography-tandem mass spectrometry (LC-MS/MS) (48). In particular, GSSG levels are influenced by oxidation during sample handling, and can appear elevated if conditions are not properly controlled (49). The glutathione redox couple ratio (GSH/GSSG) represents an efficient way to depict the intracellular antioxidant capacity, eliminating the method variability, due to the ratio measurement.

1.4 The effect of age on oxidative stress and DNA damage response

Organismal aging is a complex process that is characterized by both dysfunction of many homeostatic mechanisms and reduced durability under stressful factors. Although the biological basis of aging remains to be illustrated, many cellular dysfunctions are associated with the development of the aging phenotype, including DNA damage response (DDR) dysregulations, leading to genomic instability, mitochondrial dysfunction and cellular senescence (50). Cellular senescence, a state characterized by cell-cycle arrest in the G1 or possibly G2 phase, which prevents the proliferation of severely damaged cells, can occur either under normal circumstances, such as during embryonic development, or can be induced under cellular oxidative stress, with four different molecular mechanisms (51):

1) DDR activation by oxidative DNA damage upregulates the expression of p53 and p21/CDK-1, leading to cellular senescence (52).

2) oxidative stress activates the inhibitor of kappa-B (IκBs) kinase, increasing the action duration of the nuclear factor kappa B (NF-κB), stimulating IL-8 expression, further increasing p53 and thus leading to cellular senescence (53).

3) oxidative stress upregulates p38 mitogen-activated protein kinase (MAPK) expression leading to increased p19 expression and inducing cellular senescence (54).

4) oxidative stress increases the expression of microRNA (miRNA), inducing cellular senescence (55).

This senescent phenotype can also affect the cellular components of the immune system, leading to a phenomenon called immunosenescence. It is characterized by dysfunctional antigen recognition, reduced vaccination effectiveness and aberrations of both immune system branches, innate and adaptive immunity. These aberrations are responsible for the chronic inflammatory profile associated with the age progression. This proinflammatory phenotype, called inflammaging, is characterized by increased levels of inflammatory cytokines, i.e. Tumor Necrosis Factor – a (TNF-α), C-reactive protein (CRP), interleukin-8 (IL-8), Monocyte Chemoattractant Protein-1 (MCP1), and RANTES (Regulated on Activation, Normal T Cell Expressed and Secreted), which play a central role in the manifestation and progression of autoimmune diseases.

1.5 The effect of DNA damage response and oxidative stress in the development of systemic autoimmune diseases

The diversity of the immune system is developed in order to protect the host from the various infectious agents. However, there are two main immune aberrations, which could lead to disease. First, the there is a variety of immune deficiency syndromes, in which one or more components of the immune system are unable to protect the host effectively against a pathogen. These disorders comprise an heterogeneous group that can be divided into primary and secondary immune deficiencies (56). Primary immune deficiencies are caused by inherent dysfunction of the immune system and are chiefly genetic in cause, while secondary immune deficiencies are consequent to other underlying causes (age, immunosuppressant medication, viral infections, malnutrition, protein-losing conditions) (57).

On the other hand, the failure of the immune components to distinguish self from non-self and react to antigens, native to itself, is the basis for autoimmune diseases (58). The immune tolerance, the ability to prevent immune responses against auto-antigens, is achieved via inactivation of the self-reactive cells, that recognize and attack to their own antigens. In order to avoid this harmful self-reactivity, self-tolerance is achieved in two levels : peripheral and central. Central tolerance is achieved in the thymus and bone marrow, where lymphocytes with self-reactivity potential are negatively selected and deleted or rendered anergic. Although a great percentage of developing cells are deleted in central lymphoid organs, a small number of cells can still escape from central deletion and reach to periphery. At second level, peripheral tolerance is regulated through apoptosis, anergy and T-regulatory cell action in the secondary lymphoid organs (59).

The inability of immune tolerance and recognition of self from non-self antigens may lead to the development of autoimmunity, with deleterious effect on tissue integrity. The diseases characterized by this aberrant activation are called autoimmune diseases and can be classified into organoid and systemic autoimmune diseases (60).

The exact mechanisms that contribute to the manifestation and perpetuation of autoimmune diseases are not fully elucidated, however both oxidative stress and DNA damage Response aberrations has been shown to be involved in the pathogenesis of these diseases. For instance,

elevated oxidative stress has been shown to alter the epigenetic (histone acetylation, methylation, citrullination, ubiquitination, phosphorylation, and sumo-ylation) and transcriptomic (increased production proinflammatory cytokines) landscape in the context of many systemic autoimmune diseases, such as Systemic Lupus Erythematosus and Rheumatoid arthritis (61). Furthermore, many systemic autoimmune diseases (Systemic Lupus Erythematosus, Rheumatoid arthritis, Systemic Sclerosis) has been shown to be associated with deficiencies in the DNA damage response and repair (DDR/R) network, leading to DNA damage accumulation and thus deleterious cellular consequences (cellular senescence, apoptosis, necrosis) (62-64).

1.5.1 Systemic Lupus Erythematosus

Systemic Lupus Erythematosus (SLE) is the prototypic systemic autoimmune diseases, characterized by heterogenous clinical manifestations, ranging from mild (i.e. rash, fever, clinically quiescent serologically active SLE) to severe life-threatening complications (lupus nephritis, seizures, acute pericarditis, acute autoimmune pancytopenia) (65). Although SLE mortality has greatly declined to a 15-year survival of 85–95% in the last decade, mainly due to earlier diagnosis and more effective medical management, excessive organ-specific damage remains till today (65,66) (Fig. 4.). SLE has a clear gender tendency, predominantly affecting women of childbearing age over men with a ratio of approximately 90% (67).

Entry criterion
Anti-nuclear antibodies at a titre of ≥1:80* on HEp-2 cells or an equivalent positive test

Additive criteria
Do not count a criterion if an explanation other than systemic lupus erythematosus is more likely
Occurrence of a criterion on at least one occasion is sufficient
At least one clinical criterion is required
Criteria need not occur simultaneously
Within each domain, only the highest weighted criterion is counted toward the total score

Clinical domains and criteria	Weight	Immunological domains and criteria	Weight
Constitutional Fever	 2	**Anti-phospholipid antibodies** Anti-cardiolipin antibodies or anti-β2GP1 antibodies or lupus anticoagulant	 2
Cutaneous Non-scarring alopecia Oral ulcers Subacute cutaneous or discoid lupus Acute cutaneous lupus	 2 2 4 6	**Complement proteins** Low C3 or low C4 Low C3 and low C4	 3 4
Arthritis Either synovitis characterised by swelling or effusion in ≥two joints or tenderness in ≥two joints plus ≥30 min of morning stiffness	 6	**Highly specific antibodies** Anti-dsDNA antibody† Anti-Smith antibody	 6 6
Neurological Delirium Psychosis Seizure	 2 3 5		
Serositis Pleural or pericardial effusion Acute pericarditis	 5 6		
Haematological Leucopenia Thrombocytopenia Autoimmune haemolysis	 3 4 4		
Renal Proteinuria >0·5 g/24 h Renal biopsy class II or V lupus nephritis Renal biopsy class III or IV lupus nephritis	 4 8 10		

Classify as systemic lupus erythematosus with a score of 10 or more if entry criterion fulfilled

Figure 4*: The development of 2019 European League Against Rheumatism and American College of Rheumatology classification criteria for systemic lupus erythematosus made possible an early and precise diagnosis.*

Many genetic and environmental factors can influence the development of SLE. Genetic factors alone do not explain the development of the SLE phenotype, since monozygotic twins have an inheritance rate of about 25% but only 2% in dizygotic twins (68). Genome-wide association studies (GWAs) implicate several candidate loci including mutations in genes associated with innate immunity (*IRF5 and IRF7*), deficiencies in complement pathways (C2, C4 and C1q) and specific ancestral major histocompatibility complex (MHC) 8.1 haplotypes (HLA-B8 and HLA-DR3) (69-72). Environmental factors that have a well-described association with triggering the disease include: UV radiation, specific drugs and viral infections. The first two environmental factors mentioned may promote the pathogenesis of SLE through their effects on the genome, causing DNA damage in the form of DNA strand breaks or altering the methylation of the DNA (73). Viral infections, especially with Epstein–Barr virus (EBV), may trigger the disease, since T-cell responses in SLE are defective (74).

Although the exact pathophysiological mechanism of SLE remains unknown, it is accepted that SLE is caused by an inappropriate autoimmune reaction against nucleic acid cellular particles, orchestrated by both the innate and the adaptive immune response (69). Products of apoptotic cells, such as DNA and RNA, can act as a potential stimulant of the innate immune response, by activating TLR (Toll-like receptor) and TLR-independent nucleic acid sensors, that lead to type I interferon expression. This IFN signature, distinctive of SLE, is strongly associated with elevated production of autoantibodies containing RNA-binding proteins, such as anti-Ro and anti-La (75). Recent data show that apart from the TLR sensors non-TLR cytoplasmic sensors (RIG-1, MDA-5 and cGAS) may also play a significant role in the pathogenesis of SLE (76,77).

Oxidative stress is thought to contribute to some of the aberrant exposure and reaction to the endogenous antigens and autoantibodies, mainly produced by the increased cell-death signals, typical of the SLE pathogenesis (78). SLE patients exhibit many signs of increased formation of oxidative stress in their peripheral mononuclear cells and their T-cell populations.

In particular, T-cells from SLE patients exhibit mitochondrial hyperpolarization (increased mitochondrial transmembrane potential [$\Delta\Psi m$]), increased mitochondrial biogenesis(79) and diminished mitochondrial turnover(80), thus leading to increased production of reactive oxygen intermediates (ROI), mitochondrial dysfunction and oxidative stress (81,82). This augmentation of oxidative stress levels may mediate T-cell dysfunction, since ROI can

regulate many signal-transduction pathways in transcriptional, translational and epigenetic level, Such T-cell defects may result in production of oxidative autoantibodies and proinflammatory immune responses.

Furthermore, peripheral blood lymphocytes (PBL) have been also studied in the context of oxidative stress. PBL from SLE patients exhibit lowered glutathione levels, the main anti-oxidant system, and augmented formation of ROI, generated by NOX enzymes, a secondary oxidant system, primarily in SLE phagocytes (83-85).

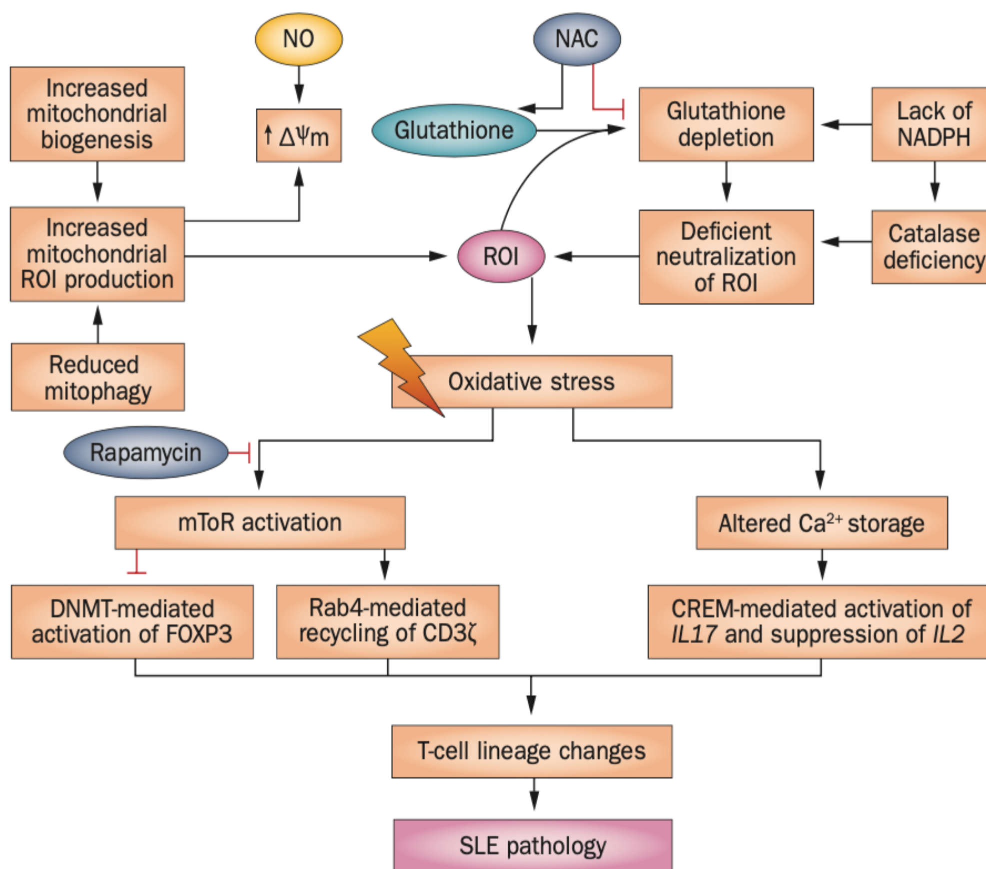


***Figure 5**: A general overview of the interplay between oxidative stress and systemic lupus erythematosus pathogenesis. Figure retrieved by Perl, A., 2013.*

Apart from augmented oxidative stress formation, DNA damage response aberrations may also be implicated in the pathogenesis of SLE. Previous studies have shown that patients with SLE display defects in many pathways implicated with DNA damage response. In particular, both nucleotide excision repair, responsible for repairing oxidative and UV-induced base lesions (86), and double-strand break repair (DSB-R), responsible for repairing the cytotoxic double-strand DNA breaks (87), exhibit lowered efficiency in the peripheral blood lymphocytes of patients with SLE, leading to intracellular accumulation of DNA damage (19,62). Though it is of interest, that patients with lower disease activity, such as patients with quiescent disease, manifest lower DNA damage levels in comparison to patients with life-threatening disease, such as patients with lupus nephritis, although both higher than the healthy individuals, suggesting an association of endogenous DNA damage levels with SLE disease activity (19).

1.5.2. Rheumatoid Arthritis

Rheumatoid arthritis (RA) is one of the most prevalent chronic inflammatory autoimmune diseases with an incidence of 0.5-1% with an apparent declining distribution from north to south and from urban to rural areas (88,89). The mean age of the average RA patient is identified to be approximately 63 years with a clear tendency towards female sex with a woman to man ratio of 4/1 (90).

Many risk factors for developing rheumatoid arthritis have been reported, including smoking (91), lower socioeconomic status (92), certain HLA haplotypes (93), particularly HLA-DRB1 (especially HLA-DRB1*01 and HLA-DRB1*04) being the most prominent, and specific epigenetic modifications (94). These risk factors can alone or cumulatively increase the risk for developing RA.

Apart from the genetic and epigenetic predisposition for developing rheumatoid arthritis, many environmental factors have also been implicated in the manifestation of RA. For example, RA has been long associated with infectious triggers by Proteus, Escherichia coli and Epstein-Barr virus. These infectious agents can lead to RA development mainly via molecular mimicry mechanisms (95,96). Furthermore, the gut microbiota plays a critical role in the development of the disease. Many animal models have shown that microbial population changes can lead to altered immune responses and arthritis exacerbation (97). Initial human studies in patients with rheumatoid arthritis have confirmed these associations (98).

Although the presence of autoantibodies against self-antigens is a main characteristic of autoimmune diseases, many cases of rheumatoid arthritis very often do not develop autoantibodies. Therefore, the disease can be divided into seropositive RA, characterized by the presence of autoantibodies, and seronegative RA, characterized by the absence of autoantibodies (88). Two distinctive types of autoantibodies are observed in the RA, the rheumatoid factor (RF), an IgM autoantibody against the Fc part of IgG antibodies, and autoantibodies against cyclic citrullinated peptides (anti-cyclic citrullinated peptides / anti-CCP autoantibodies). The rheumatoid factor is the first autoantibody observed and studied in patients with rheumatoid arthritis. However it can also be observed in patients with infectious and other autoimmune diseases, therefore its diagnostic value is very limited. On the other hand, anti-CCP autoantibodies are mainly found in RA patients (specificity= 95%). Anti-CCP

antibodies recognize many citrullinated self-proteins, including vimentine, a-enolase, fibronectin, fibrinogen and Type II collagen (88,99).

This autoantibody production in rheumatoid arthritis is mainly attributed to aberrations of both the innate and adaptive arm of the immune response in genetically pre-disposed individuals. In individuals carrying genetic risk factors, such as risk HLA-aplotypes, citrullinated peptides, produced from the aforementioned extrinsic factors, can be more efficiently presented, leading to anti-CCP autoantibodies via subsequent T- and B- cell activation. Thus, modified self-peptides are recognized by innate B-cells and are presented to autoreactive T-cells, leading to autoantibody production. These auto-reactive T-cell clones are not deleted by the thymus due to their unavailability during development. However, it is of interest that circulating anti-CCP autoantibodies can be observed up to 10 years prior active disease manifestation, a state called pre-RA (99,100).

In RA the main tissue-target is synovium. Autoantibody production is not capable to lead to synovial inflammation per se. Random tissue insults, such as viral infection or microtrauma, can lead to immune complex formation and vascular activation on the synovial area. Synovial innate immune cells (mast cells) release vaso-active mediators, further increasing auto-antibody access to self-reacting T-cells and B-cells, further overproduction of autoantibodies, innate immune cell activation (macrophages, neutrophils). Innate immune cells release pro-inflammatory cytokines (TNF, IL-1, IL-6), adhesion molecules and matrix degrading enzymes (matrix metalloproteinases). As a result, synovial hypertrophy, in the form of synovial "pannus", cartilage and bone erosion, due to increased chondrocyte catabolism and osteoclast neogenesis are observed (100-102).

These immune aberrations, observed in RA, have been shown to be associated with oxidative stress and DNA damage response deregulations. Accumulation of DNA damage in many immune cell populations in patients with RA can be a result of both increased DNA damage formation and decreased DNA damage repair (9). In the context of increased DNA damage formation, oxidative stress is the key mediator of DNA damage formation. Increased levels of reactive oxygen species and diminished antioxidant potential can be observed both in the sera and synoviums of many patients with RA. It is further supported that oxidative stress levels may be even associated with disease activity (103). As far as DNA damage repair aberrations are concerned, numerous studies have shown that many immune cell populations in RA

patients exhibit significant defects in many DNA damage repair pathways, which are associated with increased cellular senescence and apoptosis. For instance, RA peripheral blood lymphocytes are characterized by diminished repair capacity of the nucleotide excision repair pathway, controlled by the degree of chromatin condensation. Furthermore, RA T-cells has been shown to be radiation-sensitive with delayed DNA damage repair, due to downregulation of significant DNA damage sensors (ATM, Rad50, MRE11, and NBS1). This accumulation of DNA damage due to both increased DNA damage formation and decreased DNA repair capacity is thought to be significantly implicated in the pathogenesis of RA (64, 104, 105).

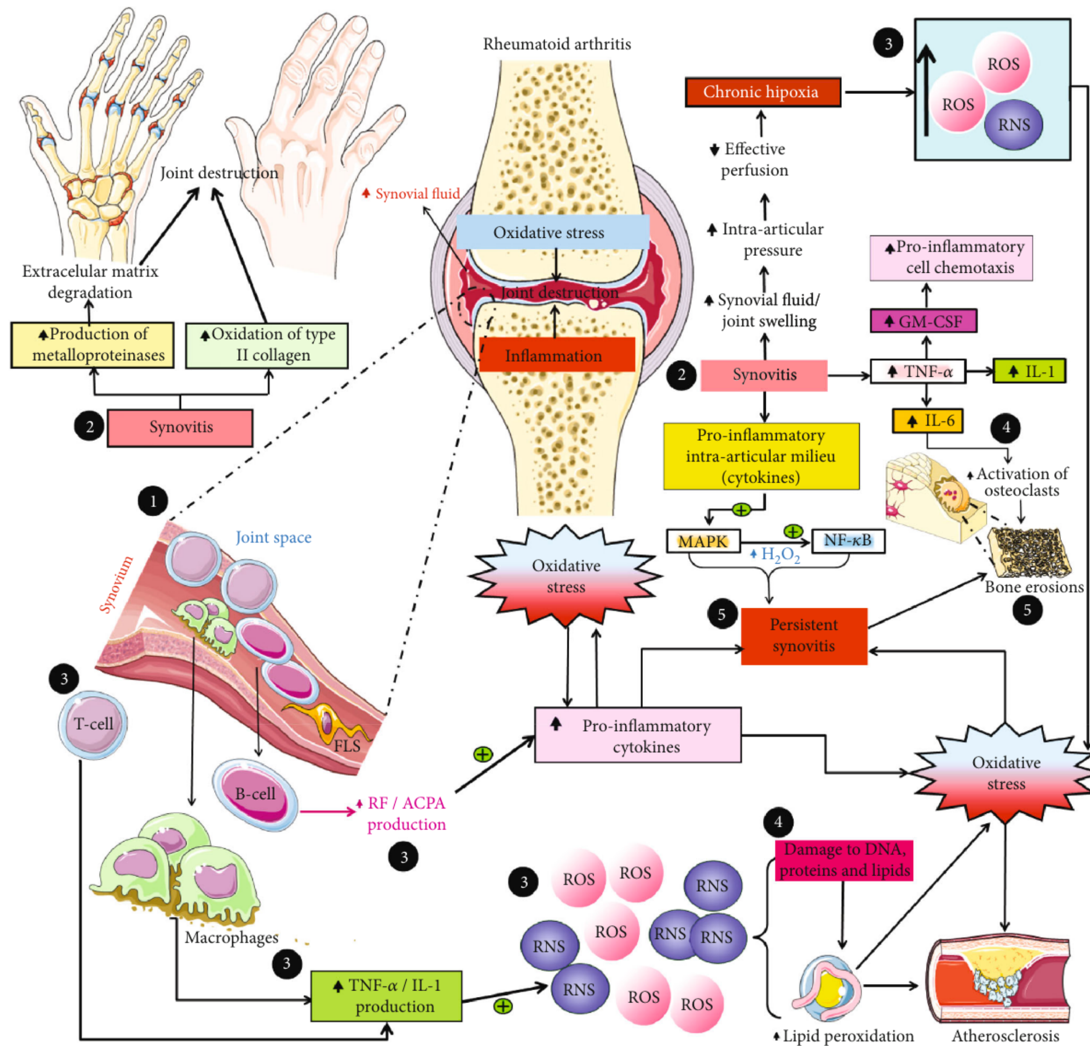


__Figure 6.__ The pathogenetic mechanisms of oxidative stress and subsequent inflammation in the patients with rheumatoid arthritis. Figure retrieved by Smallwood, M.J et. al 2018.

1.5.3 Systemic Sclerosis

Systemic sclerosis is a chronic autoimmune-driven fibrosing disease that it mainly involves the skin but also several other internal organs (lungs, kidneys, intestine, muscle). It can manifest with many clinical features that include vascular abnormalities, such as the Raynaud's phenomenon (diminished blood supply due to irregular vascular spasm causing discoloration of the extremities) and digital ulcerations, severe fibrosis of the skin of the extremities and the face, pulmonary fibrosis, renal failure, pulmonary arterial hypertension or gastrointestinal complications, such as gastrointestinal reflux (106-108).

Due to the great clinical heterogeneity of the disease two subsets have been accepted: limited and diffuse cutaneous systemic sclerosis (lc-SSc and dc-SSc respectively), with the only difference being in the speed of the disease progression and the extent of skin and visceral involvement. In limited disease, the fibrosis is restricted only in the distal skin of the extremities and is frequently associated with late-stage only serious complications. On the other hand, in diffuse cutaneous disease, not only the proximal skin of the limb but also the trunk are affected, while this subtype of the disease is also associated with higher frequency cardiac involvement and renal crisis, a life-threatening complication consisting of thrombotic microangiopathy, hypertension and progressive acute kidney injury (107,109,110). Another hallmark of the disease is the production of specific autoantibodies, including anti-centromere, anti-topoisomerase I and anti-RNA polymerase III antibodies (107,111). If systemic sclerosis is suspected, a definite diagnosis should be ascertained, using the 2013 European League Against Rheumatism (EULAR) and American College of Rheumatology (ACR) classification criteria (112,113).

2013 American College of Rheumatology and European League Against Rheumatism criteria for the classification of systemic sclerosis *A total score of 9 is needed for a definite classification.
1. Proximal skin involvement
Skin thickening of the fingers of both hands, extending proximal to the metacarpophalangeal joints (sufficient criterion; score 9)
2. Skin thickening of the fingers (only count the higher score)
a) Puffy fingers (score 2) b) Sclerodactyly of the fingers (distal to the metacarpophalangeal joints but proximal to the proximal interphalangeal joints; score 4)
3. Fingertip lesions (only count the higher score)
a) Digital tip ulcers (score 2) b) Fingertip pitting scars (score 3)
4. Telangiectasia (score 2)
5. Abnormal nailfold capillaries (score 2)
6. Pulmonary arterial hypertension or interstitial lung disease (maximum score of 2)
a) Pulmonary arterial hypertension (score 2) b) Interstitial lung disease (score 2)
7. Raynaud's phenomenon (score 3)
8. Systemic sclerosis-related autoantibodies (maximum score of 3)
a) Anti-centromere (score 3) b) Anti-topoisomeraseI (score 3) c) Anti-RNA polymerase III (score 3)

Table 1. *Summary items from the 2013 American College of Rheumatology and European League Against Rheumatism criteria for the classification of systemic sclerosis*

Many risks factors have been associated with the development of Systemic Sclerosis including sex hormones, genetic predisposition and environmental encumbrance. The disease mostly affects adult females, with a reported 6:1 female to male ratio by the EUSTAR registry (114). The role of female sex hormones in the fibrosis development is supported by the modulatory effects of oestrogens on extracellular matrix synthesis and the expression of adhesion molecules in patients with systemic sclerosis (115). In genetic level, studies have shown that SSc heritability is present, although lower than other autoimmune diseases and a positive family history of SSc is a strong predictor for SSc development. Moreover, many large-scale studies indicated that HLA-DQB1 haplotype (HLA-DPB1*13:01, HLA-DQB1*05:01, HLADRB1*04:04, HLA-DRB1*11, and HLA-DQB1*03) is strongly associated with SSc susceptibility (116-119). Many studies focusing on the environmental etiology of systemic sclerosis has identified many environmental agents related to the disease manifestation, such as vinyl chloride, silica, epoxy resins, rapeseed oil and certain drugs (Bleomycin, Carbidopa, L-5-hydroxytryptophan, Pentazocine, Cocaine) (120-123).

Even though the pathophysiology of Systemic Sclerosis remains mainly unknown, vasculopathy is suspected to be a major trigger for the aberrant immune responses observed. Damaged or apoptotic endothelial cells release damage-associated molecular patterns (DAMPs) that activate the neighboring and circulating immune cells. However, irregular expression of adhesion molecules can accelerate the immune cell infiltration even before endothelial damage (124-126). Immune cells in SSc secrete increased amounts of pro-fibrotic factors, such as IL-4, IL-13 and transforming growth factor - β (TGF-β), stimulating B-cell proliferation, immunoglobulin production and extracellular matrix (ECM) differentiation. The activated fibroblasts, responsible for the ECM differentiation, have the characteristics of myofibroblasts and seem to play a key role in disease pathophysiology (127-129).

Furthermore, it is supported that oxidative stress is implicated in development of this disease. Indeed, many oxidative stress markers has been found to be significantly elevated in patients with systemic sclerosis. In particular, increased levels of malondialdehyde (MDA), lipid hydroperoxides and 8-isoprostane are observed in the sera of SSc patients (130). The total antioxidant capacity (TAC), representing the tissue ability to counteract oxidative stress, has also been reported diminished. This decrease could be attributed to the chronic inflammation status, pathological conditions, such as tissue ischemia, or gastrointestinal abnormalities (dysmotility, decreased pancreatic function, bacterial overgrowth and malabsorption of fat).

Oxidative stress mediators (ROS, RNS etc.) can stimulate the production of pro-inflammatory and pro-fibrotic cytokines, such as TGF-b and PDGF, to induce fibroblast activation (130-132)

Moreover, increased DNA damage levels have been observed in patients with systemic sclerosis, regardless of disease subtype and medication. This DNA damage accumulation is associated with specific gene polymorphisms (*XRCC1* and *XRCC4*), related to DNA damage repair pathways. These results indicate that SSc immune cells may be intrinsically predisposed for a profibrotic and proinflammatory expression profile (9,63).

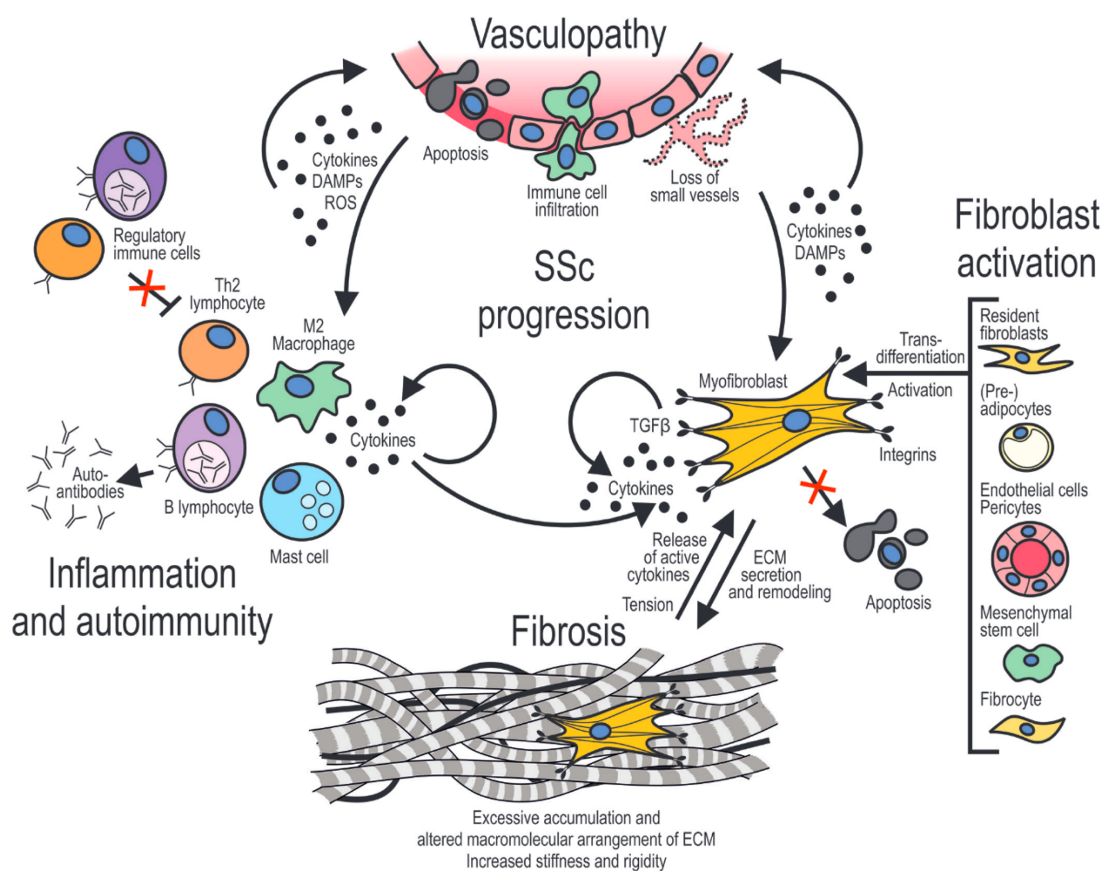


Figure 7. *The interplay between vasculopathy, autoimmunity and fibrosis is the basis of SSc pathophysiology. Figure retrieved by Rosendahl, A.-H.et. al., 2022.*

1.5.4 Adamantiades – Behcet's Disease

Adamantiades – Behcet's Disease (ABD) is a chronic recurrent multisystemic inflammatory disorder, characterized by a wide variety of clinical manifestations, including recurrent oral and genital ulcers, ocular involvement, skin lesions, arthritis, vasculitis, epididymitis, gastrointestinal and nervous system lesions (133). The disease exhibits a unique epidemiological distribution that extends along the ancient Silk Road, from the Mediterranean and the Middle Eastern countries to Far East Asian countries. Sex distribution in ABD appears to be equal among male and female individuals, although there are many geographical discrepancies, manifesting both male (Middle East) and female (Japan and Korea) predominance (134).

The pathogenesis of ABD remains mostly unknown, but genetic predisposition, environmental factors and immunological abnormalities contribute to ABD development. ABD is strongly associated with the presence of HLA-B51* allele. Two subtypes of this allele, HLA-B*5101 and HLA-B*5108, have been especially implicated with disease development(135). Recent studies have also reported additional independent associations with several MHC Class I regions, including HLA-B*15, HLA-B*27, HLA-B*57 and HLA-A*26, while HLA-B*49 and HLA-A*03 seems to gain protection against ABD development (136). Beyond the MHC genes, non-MHC regions that are implicated with ABD development include genes associated with cytokine and growth factor production (IL-1b, IL-6, IL-8, IL-10, IL-12, IL-17, IL-18, IL-23, TGF-β) (137). Previous studies have, also, shown that genes encoding proteins involved with oxidative stress (glutathione transferase and myeloperoxidase) can also be associated with increased risk for ABD development. Recent whole-exome analysis in ABD patients revealed a novel deleterious genetic variant of *NEIL-1*, a gene implicated in Base excision repair (BER) pathway, a critical pathway for the repair of oxidative DNA damage (135,138).

It has been long suggested that ABD could be possibly triggered by specific environmental (contagious or not) factors in genetically susceptible individuals. Herpes simplex virus (HSV) type 1 genome was found in peripheral blood lymphocytes, saliva fluid and genital/gastrointestinal ulcers of ABD patients. ABD patients can also exhibit increased skin reactivity against certain Streptococcus Sanguis antigens (139,140).

ABD is defined as a disorder at the intersection of autoimmunity and autoinflammation. The immunological characteristics that imply an auto-inflammatory role of ABD include aberrations in the innate immune system. This is suggested by the ABD-specific pathergy test, revealing an increased cutaneous inflammatory response, due to innate immune response exaggerations. Pathogen recognition receptors (PRR), components of the innate immune response, consist of the pathogen associated molecular patterns (PAMP) and the damage associated molecular patterns (DAMP), exhibit increased expression and activation in patients with ABD (141). This activation can result in cytokine (IL-1b and IL-18) expression via the NF-kb pathway and the NLRP3-inflammasome activation. In ABD patients, NLRP3-inflamasome is reported to be increased in transcriptional and translational level, with an increased IL-1b response after stimulation (142). Neutrophil hyperactivation is also an essential part of the auto-inflammatory pathogenesis of ABD, with neutrophil activation markers, such as CD64, being significantly increased in ABD patients (143).

There are major characteristics that can classify ABD in the spectrum of autoimmune diseases. First of all, ABD exhibits a strong genetic predisposal associated with the presence of the HLA-B51 haplotype, a common feature with other autoimmune diseases (136). Moreover the pathogenetic role of aberrations on the adaptive arm of immune response, subsets of T cells, especially Th17, playing a crucial role in ABD development, is a major feature that connects ABD to other autoimmune diseases (144,145). Finally, the successful disease control with the conventional immunosuppressive agents, such as cyclosporine and cyclophosphamide underlie the autoimmune character of ABD (146).

The autoinflammatory status observed in ABD can be partly attributed to oxidative stress aberrations in essential immune response cellular components. Previous studies have associated an oxidative stress status, mainly observed in the neutrophiles of ABD patients, with increased chemoattraction, phagocytosis and free radical secretion (147). There is an imbalance of the oxidative cellular stratus consisting of a significant increase of the oxidant pathways combined with a diminished antioxidant capacity (148,149). This oxidant excess could be also connected with the increased thrombotic risk, a severe complication of ABD. Activated neutrophiles manifest a pro-oxidant profile, secreting great amounts of reactive oxygen species, could alter the secondary structure of fibrinogen, thus connecting the oxidative stress with the thrombo-inflammatory cascade in ABD (150,151).

1.5.5 Antiphospholipid Syndrome

Antiphospholipid syndrome (APS) is a systemic autoimmune disease, which is characterized by arterial, venous or small vessel thrombosis, the persistent presence of positive antiphospholipid antibodies (aPL) and recurrent obstetric complications, including early pregnancy loss, fetal loss, or pregnancy morbidity, despite a sufficient antiplatelet/anticoagulation treatment (152,153). APL antibodies may include three different antiphospholipid antibodies: lupus anticoagulant; anticardiolipin; and anti-beta 2 glycoprotein I. The most recent APS classifying criteria require the persistent presence of anticardiolipin antibodies or anti-beta-2 glycoprotein I antibodies (anti-β2GPI), combined with at least one thrombotic or obstetric morbidity event (154).

APS can manifest in many forms: firstly the primary form (primary APS, pAPS), occurring as an isolated, without any other background associated condition, or in association with other systemic autoimmune diseases, mainly systemic lupus erythematosus, named secondary APS (sAPS). Although both types of APS can exhibit similar clinical and biological features, there is a trend of prevalence of specific features in every disease subtype (155,156). However, both APS types are associated with increased morbidity and mortality compared to the general population. This is mainly attributed to the increased thrombotic complications, affecting both arteries and venous of critical organs (brain, heart, lungs, and kidneys) (157). Therefore, the presence of APL and specific clinical signs can categorize APS in specific subtypes: "classical/thrombotic" APS, involving recurrent vascular events, such as venous and/or arterial thrombosis, APS limited to adverse obstetric events (obstetric APS), asymptomatic APL positive carrier individuals, patients with life-threatening disease symptoms (catastrophic APS/ cAPS)(158).

Although the exact etiology of APS remains unknown, it is theorized to be multifactorial, consisting of genetic, environmental and immunological components. The genetic basis in the development of APS is supported by the development of clinical features in monozygotic twins. Specific genetic variants have also been connected to the pathogenesis of APS (159,160). Different HLA haplotypes has been associated with PAPS and SAPS, in particular the HLA-DR7 for the PAPS and the HLA-B8, HLA-DR2, HLA-DR3 for the secondary APS (161,162). Apart from the HLA-associated genes, many genetic studies have unraveled genes outside the HLA area that could contribute to disease susceptibility, clinical heterogeneity, and

autoantibody production in APS. The first genetic non-HLA risk factor for APS development is associated with polymorphisms on the B2-GPI, leading to an amino-acid change and correlating with anti-B2GPI production and reactivity (163). Furthermore, genetic polymorphisms in the STAT4, a key gene encoding signal transducer and activator of transcription-4 (STAT4), considered a common genetic risk factor for multiple autoimmune diseases, are associated with APS development (164).

Infections are considered one of the most prominent environmental factors for APS development. Historically, Treponema pallidum, responsible for syphilis, was the first infectious agent to be associated with APS, since cardiolipins were the major tissue extract used in the Venereal Disease Research Laboratory (VDRL) test, used for its diagnosis (165). Over recent years it has been reported that many bacterial and viral infections can not only lead to APL antibodies' production but also to clinical signs of APS. These infections include Treponema pallidum, leishmaniasis, Leptospiara interrogans, Neisseria, Plasmodium species, Streptococcus Pyogenes, Helicobacter pylori, Mycoplasma pneumoniae, Hepatitis virus (A-D), Varicella Zoster Virus (VZV), Epstein-Barr Virus (EBV), Cytomegalovirus (CMV) and several adenovirus species (166-171). Furthermore, specific medications have been associated with APL manifestation. These medications include amoxicillin, antiepileptic drugs (phenytoin, valproate, ethosuximide), anti-hypertensive agents (chlorothiazide, propanolol, hydralazine), oral contraceptives, phenothiazines (especially chlorpromazine), antiarhthmics (procainamide, quinidine, quinine) and immunomodulatory drugs such as alpha interferon and infliximab (172,173).

Although the exact pathogenetic mechanism, which makes an individual prone to APS manifestation, remains to be elucidated, it is accepted that the pathogenesis of APS consists of two steps, the first and second hit (158,174). The first hit is represented by the presence of APL antibodies and their interference with endothelium, inducing the procoagulant phenotype. The APL family consists of an heterogenous group of antibodies directed against phospholipid-binding proteins, β2-glycoprotein I (β2GPI) being the most characteristic. Other PL-binding proteins include prothrombin, thrombomodulin, antithrombin III, protein C and S and annexin I,II and V. Anti-β2GPI can interact with monocytes and endothelial cells leading to tissue factor upregulation and expression of adhesion molecules, such as intercellular cell adhesion molecule-1 (ICAM-I) and vascular cell adhesion molecule-1 (VCAM-I), inducing a

proinflammatory and procoagulant phenotype. Moreover, APL can lead to platelet activation, increasing the secretion of procoagulant and prothrombotic chemokines, such as glycoprotein II b-IIIa, thromboxane A2 and platelet factor-IV (175).

However, it is likely that APL presence is a necessary but not sufficient condition to trigger the disease activation. Therefore a second hit may be necessary. This second hit, acting as triggering factor includes acute infections, trauma and smoking, leading to inflammation and increased oxidative stress development. Several mechanisms have been proposed as promoters of oxidative stress in APS patients. Oxidative stress can be induced by increase in the mitochondrial transmembrane potential, decrease of intracellular antioxidant enzymes, such as the intracellular glutathione concentration and the paraoxonase-1 (PON-1) expression, and increase of pro-oxidant molecule production, such as nitric oxide (NO), superoxide (O2−) and peroxynitrite (ONOO−) (176).

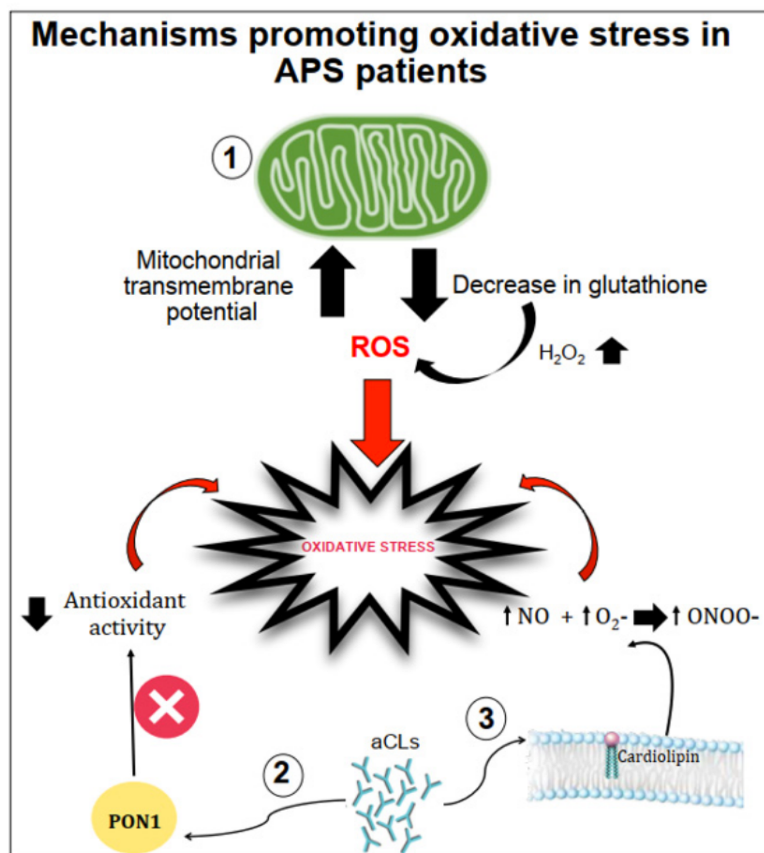


Figure 8: *The mechanisms promoting oxidative stress in patients with APS. Figure retrieved by Nocella, C. et. al 2021.*

EXPERIMENTAL AND CLINICAL DATA

(ΕΙΔΙΚΟ ΜΕΡΟΣ)

2. Rationale and Purpose of the study

Systemic autoimmune diseases include a group of diverse diseases which are characterized by chronic aberrant immune activation against self-antigens, extensive production of autoantibodies and ultimately tissue injury. Pathophysiologically, oxidative stress, the imbalance between the oxidant and antioxidant cellular systems after exposure to deleterious stimuli, is shown to play a significant role in the manifestation and progression of these diseases. Reactive oxygen and nitrogen species (ROS and RNS respectively), key representators of oxidative stress, can alter the structure of intracellular macromolecules, such as DNA, leading to oxidative damage.

However, the genome is protected by a well-organized mechanism, the DDR/R network, minimizing the effect of those insults. Recent studies suggest that systemic autoimmune diseases may manifest defects in both the oxidative stress regulation, generating higher levels of oxidative DNA damage, and the DNA damage response network, thus further accumulating DNA damage. This intracellular DNA damage accumulation can result in cytosolic DNA fragments accumulation which may act as potent immunostimulators, further propagating disease progress.

Finally, aging, a complex biological process recognized by the progressive deregulation of the homeostatic mechanisms and reduced durability to stress, has been also associated with deregulations on oxidative stress and DNA damage responses. Many distinguishing features of the aging phenotype, such as genomic instability, mitochondrial dysfunction and cellular senescence may be a result of defects on oxidative stress and DDR. Systemic autoimmune diseases manifest a similar phenotype, although the affected population mainly includes young and middle-aged adults.

Based upon these data, herein we investigated:

1) whether patients with systemic autoimmune diseases manifest augmented oxidative stress

2) whether patients with systemic autoimmune diseases display increased accumulation of DNA damage

3) whether organismal aging contributes to the pathophysiology of systemic autoimmunity

3. Study population and methodology

3.1 Study population

Seventy eight (N=78) consecutive adult patients, attending the rheumatology outpatient clinic of the first department of propaedeutic and internal medicine of Laiko General Hospital, were recruited between February 2019 and June 2023. In particular, the patients participating in the study may be diagnosed:

- With Rheumatoid Arthritis, meeting the 2010 ACR/EULAR classification criteria for Rheumatoid arthritis.
- With Systemic Lupus Erythematosus, meeting the 2019 ACR/EULAR classification criteria for Systemic Lupus Erythematosus.
- With Systemic Sclerosis, meeting the 2013 EULAR/ACR classification criteria for Systemic Sclerosis.
- With Adamatiades'-Behcet's disease, meeting the 2006 International Criteria for Behcet's Disease.
- With Antiphospholipid Syndrome, meeting the updated Sapporo classification criteria for Antiphospholipid Syndrome.

Furthermore, 212 apparently healthy individuals served as controls (HC). Exclusion criteria included:

- personal or family history of systemic autoimmunity
- past or current history of malignancy
- active or recent (last 2 weeks) infection

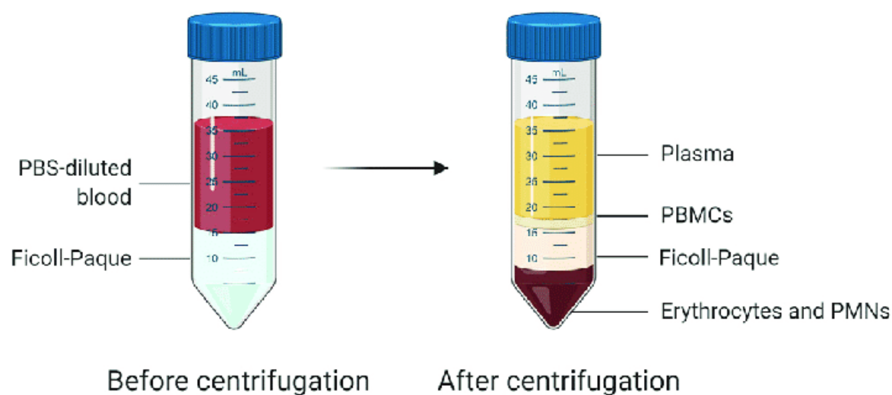
At the time of the recruitment, demographics and disease characteristics of each individual were noted. The disease characteristics included patients' age, gender, smoking habits, disease duration, clinical signs, laboratory parameters (ESR, CRP, autoantibody status), disease activity indices and medication at the time of the sampling.

The study was approved by "Laiko" Hospital Ethical Committee (No 1110) and all participants gave written informed consent in compliance with the Helsinki Declaration.

3.2 Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were isolated and purified using the standard Ficoll gradient centrifugation method. The isolation and collection of PBMCs was performed immediately after the venipuncture of each individual.

At first, approximately 20ml of peripheral blood was collected in EDTA tubes. The peripheral blood was afterwards diluted 1/1 with 1xPBS (Phosphate Buffer Saline; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4) in 15ml sterile falcon tubes. Then, the diluted peripheral blood is carefully placed in sterile falcon tubes with Ficoll density gradient medium (Ficoll-PaquePlus, Cytiva, Sigma-Aldrich). The PBMCs are isolated by Ficoll density gradient centrifugation at 400g for 30 minutes in room temperature (20° C). After the centrifugation, the upper layer, which mainly consists of plasma, is discarded. The mesophase is carefully transferred into a new 15ml sterile falcon tube, without aspirating the lower layer, which mainly consists of Ficoll and higher density cells, such as neutrophils.



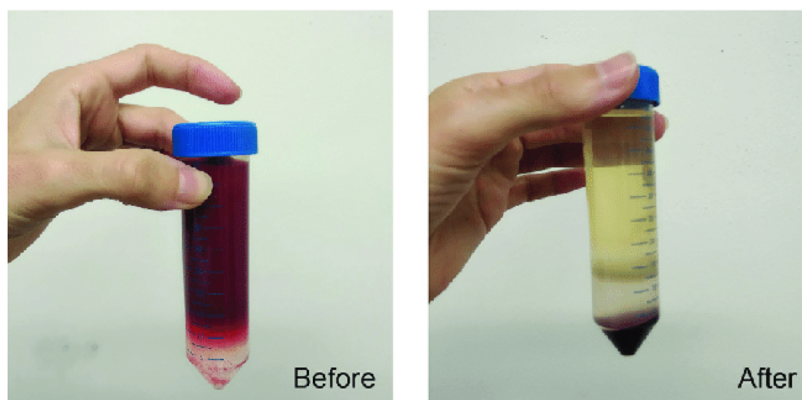


Figure 9: *Illustration of the Ficoll density gradient centrifugation during peripheral mononuclear cell isolation.*

The PBMC layer is washed in 15ml 1x PBS and centrifuged at 200g for 10 minutes. Afterwards, PBMCs are washed a second time using 10ml 1x PBS and again centrifuged at 200g for 10 minutes. The cellular composition of the final cell pellet is controlled using Flow cytometry. Finally, the cell pellet is stored in Freezing Medium (90% Fetal Bovine Serum [FBS] + 10% Dimethylsulfoxide [DMSO]) and stored at -80°C for further use. Another smaller part of the cell pellet is lysed in TRIzol® (Invitrogen, ThermoFisher Scientific) and stored at -80°C for further use.

3.2 DNA damage measurement by alkaline comet assay

The single-cell gel electrophoresis under alkaline conditions (alkaline comet assay) can quantify single- and double- strand DNA breaks (SSBs and DSBs respectively) with high sensitivity, being able to measure a range of strand breaks between 50-10.000 DNA strand breaks per cell. The process of alkaline comet assay can be divided in distinctive steps.

1. The first step includes the dissolution of the low-melting agarose in 1x PBS solution at 100°C. Special pre-coated slides with agarose must be obtained before the start of the experiment. These pre-coated slides can be either purchased by Trevigen® (CometAssay HT Slide, Trevigen, USA) or prepared several days ahead, to be ensured that they have dried. PBMCs are progressively defrosted, resuspended in the 1% low-melting agarose-PBS solution at 37°C and spread onto the pre-coated slides.

2. The slides stay at 4°C for 30 minutes, in order for the agarose gel to be fixated.

3. Subsequently, the slides are placed in a sterile lysis buffer at 4°C for 2 hours, consisting of 2.5M NaCl, 0.1M EDTA, 10mM Tris (pH=10) and 1% Triton X-100, dissolving the cellular membranes.

4. Then the slides are placed in an horizontal gel electrophoresis chamber, first incubated in pre-chilled electrophoresis buffer (0.3M NaOH, 1mM EDTA) for 40 minutes at 4°C without electric current and then for 30 minutes at 1 V/cm.

5. Afterwards, the slides are washed in sterile neutralizing buffer (0.4M Tris - HCl, pH=7.5) for 30 minutes and double-distilled water for another 30 minutes.

6. Finally, the slides are saved in a dark place in room temperature and left to dry overnight.

7. The gels are stained using the SYBR Gold Nucleic Acid Gel Stain (Thermo Fischer Scientific) and left under no light conditions for approximately 30 minutes.

8. The samples are observed and analyzed, using a fluorescence microscope (Zeiss Axiophot)

9. DNA damage is evaluated, using the Olive Tail Moment [OTM = (Tail Mean-Head Mean) x (% of DNA)/100] of at least 200 cells per sample. The parameter analysis is achieved by the ImageJ Analysis/Open Comet software.

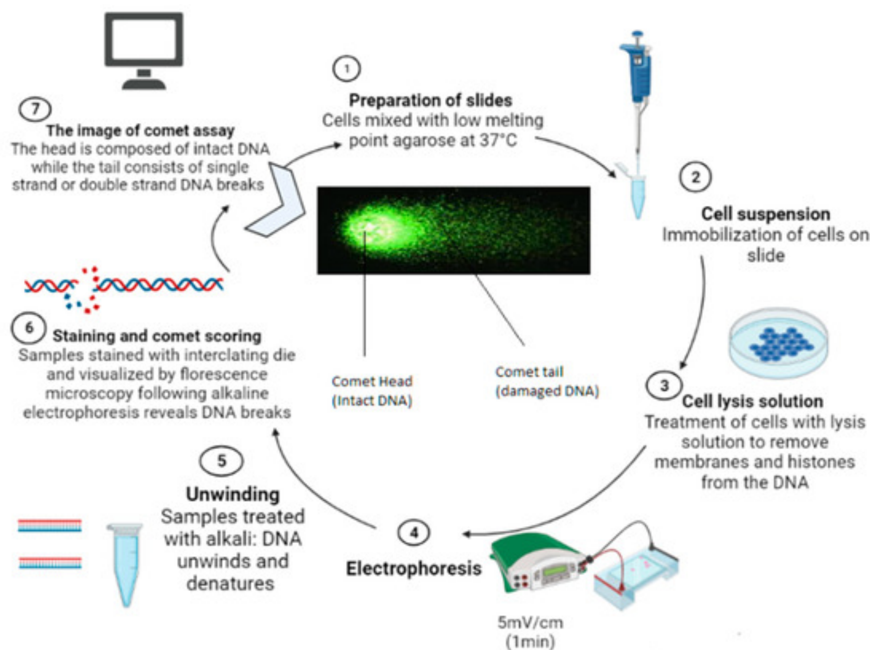


Figure 10: *Illustration of the multi-step process of alkaline comet assay.*

3.3 Oxidative stress quantification by measurement of glutathione (GSH) and oxidized glutathione (GSSG)

Basal intracellular oxidative stress was assessed using a luminescence-based system that detects and measures total glutathione (GSH+GSSG), oxidized glutathione (GSSG) and the reduced to oxidized glutathione ratio (GSH/GSSG), according to manufacturer's protocol (GSH/GSSG-Glo™ Assay, Promega).

The quantification of total and oxidized glutathione is based on a chemical reaction, where GSH-dependent conversion of a GSH probe, Luciferin-NT, to luciferin by a glutathione-S-transferase enzyme is coupled to a firefly luciferase reaction. The intensity of the light from luciferase is dependent on the amount of the formed luciferin, which is in turn dependent on the amount of GSH present. The total amount of glutathione is quantified, using a reducing agent that converts all the intracellular glutathione to the reduced form, GSH. The oxidized glutathione is measured by adding a second assay reagent that blocks all the GSH while leaving the GSSG intact.

The experimental protocol includes plating 10^4 PBMCs in a sterile luminometer-compatible tissue culture 96-well plate (Corning Costar). Then 50 µl/well of Luciferin Generation Reagent was added to all wells, followed by a brief shake and incubation at room temperature (25ºC) for 30 minutes. Next, 100 µl/well of Luciferin Detection Reagent were added and left at room temperature for another 15 minutes. The luminescence signal was read in a Spectramax M3 microplate reader (Molecular Devices LLC, California).

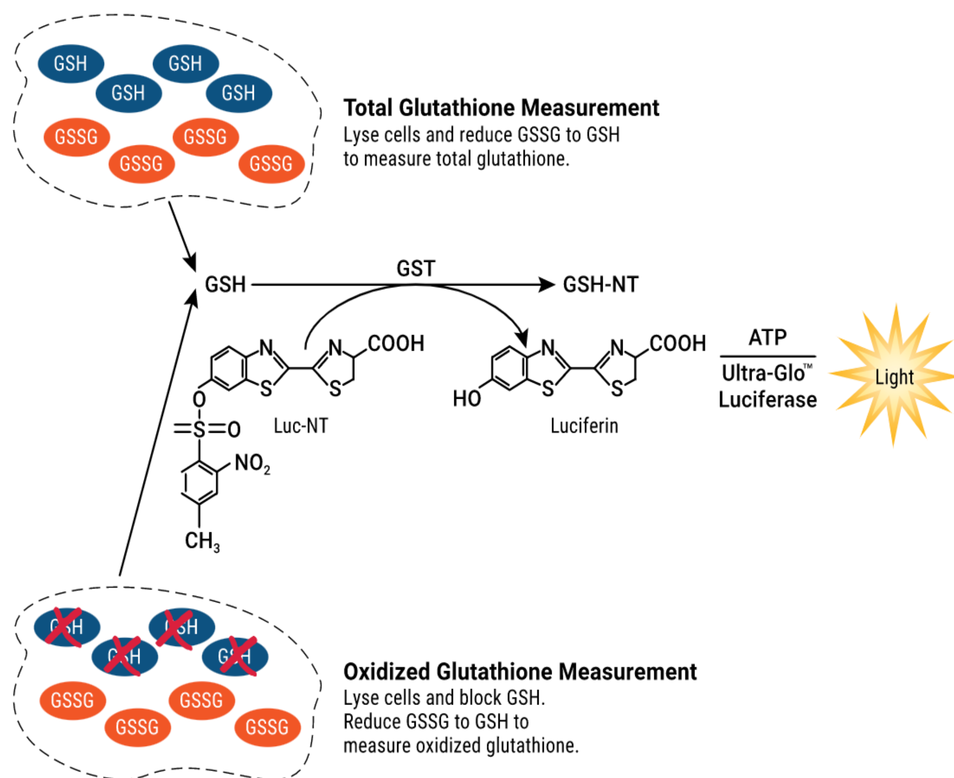


Figure 11: *Illustration of the glutathione-dependent conversion of Luciferin-NT to luciferin by a glutathione S-transferase enzyme, which is coupled to a firefly luciferase reaction. This reaction is the basic process on the oxidative stress quantification assay.*

3.4 Abasic site quantification

Among numerous types of oxidative DNA damage, apurinic/apyrimidinic sites (AP or abasic sites) are one of the most characteristic lesions of oxidative DNA damage. Abasic sites are produced in DNA at a significant rate by spontaneous base loss as in depurination during DNA oxidation. Every day is estimated that each mammalian cell encounters 50,000 to 200,000 abasic site lesions. The unrepaired lesions can inhibit the function of topoisomerases, the replication, and can lead to mutations, due to the bypass synthesis on the non-templated DNA strand.

The OxiSelect^{TM} Oxidative DNA Damage Quantitation Kit (Cell Biolabs, Inc.) can specifically evaluate the AP-sites. The kit uses an Aldehyde Reactive Probe (ARP) to react specifically with an aldehyde group on the open ring form of AP sites, allowing the AP-sites to be tagged with biotin and later allowing the detection using a Streptavidin-Enzyme conjugate. The quantity of AP sites in an unknown DNA sample is determined by comparing its absorbance with a standard curve generated from the provided DNA standard containing predetermined AP sites. The absorbance of each microplate is achieved in a Spectramax M3 microplate reader (Molecular Devices LLC, California) using the 450 nm as the primary wave length.

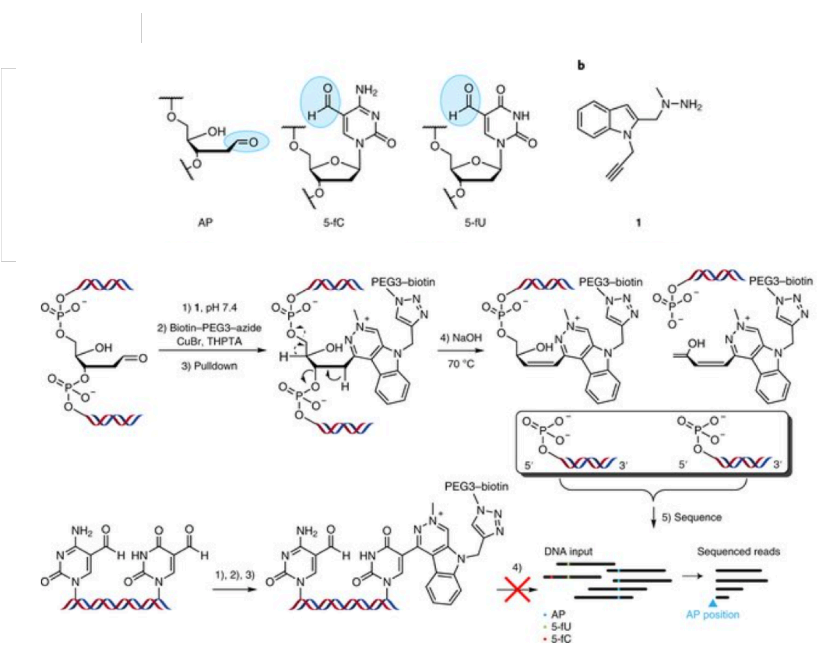


Figure 12: *Chemical tagging of AP-sites using a biotin based probe during AP-site quantification process.*

3.5 Study of double-strand DNA break repair mechanism (DSB-R)

The efficiency of the double-strand DNA breaks repair (DSB-R) mechanism was assessed by studying the phosphorylation of the histone H2AX. Following the formation of double-strand DNA breaks (DSBs) caused by ionizing radiation, UV-light or radiomimetic agents, PI3K-like kinases, including ATM, ATR, and DNA-PK, result in the phosphorylation of the histones H2AX at Ser139 along with the activation of the protein p53 through phosphorylation at Ser20. Therefore, this newly phosphorylated γH2AX histone is one of the most sensitive markers of double-strand DNA breaks, since it matches 1:1 with every DSB. Furthermore, the rate of γH2AX removal can be used to study the double-strand repair mechanism efficiency.

In this study we studied the DSB-R mechanism efficiency through its induction by melphalan, an alkylating chemotherapeutic agent, causing double-strand DNA breaks and γH2AX induction. γH2AX was quantified by immunofluorescence antigen staining and confocal laser microscopy.

Freshly isolated PBMCs were incubated in complete RPMI medium, supplemented with 10% FBS, 50 mg/l penicillin, 50,000 IU/l streptomycin and 2 mmol/l L-glutamine, and subsequently were treated with 100 μg/ml melphalan for 5 min at 37°C. Afterwards, PBMCs were incubated in drug-free medium for specific times (0-24h). Then the cells were adhered to a coverslip, coated with 1M HCI and 50 mg/ml poly-D-lysine prior to use, fixed by adding a 4% paraformaldehyde solution for six minutes at room temperature and stored at 80°C before anti-γH2AX analysis.

PBMCs were washed with cold PBS and blocked using 0.5 ml/well blocking buffer (0.1% Triton X-100, 0.2% skimmed dry milk in PBS) for 1h at 25°C in a humidified box. Blocked PBMCs were then incubated with anti- γH2AX (Phospho-Histone H2AX [Ser139] Antibody, Cell Signaling) at a dilution of 1:400 in blocking buffer at 4°C overnight.

After washing with blocking buffer, cells were incubated with goat anti-mouse antibody, fluorescein isothiocyanate (FITC) labeled or goat anti-rabbit IgG tetramethylrhodamine (TRITC) labeled (Invitrogen) at a dilution of 1:4000 in blocking buffer for one hour at room temperature in the dark. Coverslips were washed with PBS three times and then mounted with mounting medium (Vectashield G-1200). The images were retrieved using a confocal laser scanning microscope (Leica TCS SP-1). The results are expressed as the percentage of γH2AX-positive cells (having more than five foci per cell).

3.6 Statistical analysis

The variable distribution was examined by D'Agostino-Pearson and Shapiro-Wilk tests. Continuous variables are presented as mean ± SD. Continuous variables were compared among groups with Student's T-test. Independent comparisons were performed with the use of Mann-Whiney U test and paired comparisons were performed with the use of Wilcoxon signed- rank test. Differences in categorical variables were examined by chi-square test. The Kruskal-Wallis test was used for group wise differences among more than 2 groups. Correlations were examined with the use of Pearson's correlation coefficient, in case of parametric variables, or the non-parametric Spearman's test. Results were considered significant when $p < 0.05$. Statistical analysis was performed in SPSS v.26 and SigmaPlot v.14.5 (IBM, USA) and GraphPad Prism v.9.1.1 (GraphPad, USA).

4. Results

4.1 Study population demographics and clinical characteristics

In general, 78 patients and 212 healthy individuals were engaged in the study, fulfilling the aforementioned criteria. The demographical characteristics of each particular subgroup in the study are presented below:

- 9 patients with Rheumatoid Arthritis (RA), 61.3% of female sex with mean age 63.33 ± 10.98.
- 14 patients with Systemic Lupus Erythematosus (SLE), 92.9% of female sex with mean age 40.21 ± 13.75.
- 9 patients with Systemic Sclerosis (SSc), 100% of female sex with mean age 48.67 ± 17.02.
- 6 patients with Adamantiades – Behcet's disease (ABD), 100% of male sex with mean age 34.17 ± 6.401.
- 40 patients with Antiphospholipid Syndrome (APS), 72.5% of female sex with mean age 47.7 ± 13.18.

As expected, a female predominance in patients with systemic autoimmune diseases is observed, apart from the patients with ABD, where as per the rest of bibliography a male predominance is observed (177). As far as the mean age of each subgroup is concerned, the youngest patients were observed in the ABD and SLE subgroup, with a mean age of 34.17 ± 6.401 and 40.21 ± 13.75 respectively, while the oldest patients were observed in the RA subgroup with a mean age of 63.33 ± 10.98, findings that are in line with each disease epidemiological data (177-181).

A conclusive list with demographical characteristics of each subgroup is presented in the table below.

Demographics	**HC (N=212)**	**RA (N=9)**	**SLE (N=14)**	**SSc (N=9)**	**APS (N=40)**	**ABD (N=6)**
Age (mean years ± SD)	49.54 ± 20.96	63.63 ± 10.98	40.21 ± 13.75	48.67 ± 17.02	47.7 ± 13.18	34.17 ± 6.4
Sex (female gender/%)	130 /61.3%	6 / 66.7%	13 / 92.9%	9 / 100.%	29 / 72.5%	0 / 0%
disease duration (mean years ± SD)	n/a	8.75 ± 15.89	6.67 ± 9.63	3.83 ± 6.75	12 ± 8.8	4.8 ± 7.5

Table 2: *Demographical characteristics of the healthy individuals and each patient subgroup.*

- A table with the disease characteristics of the RA patients is presented below:

Disease characteristics	**DAS-28-ESR (mean ± SD)**	**Tender Joint Count (mean ± SD)**	**Swollen Joint Count (mean ± SD)**	**RF positivity (n / %)**	**Anti-CCP positivity (n / %)**	**ESR (mean± SD)**
RA (N=9)	5.23 ± 0.9	6.5 ± 4	5.1 ± 4	3 / 37.5 %	6 / 75.0%	35 ±15

Table 3: *Clinical and laboratory characteristics of the RA subgroup.*

- A table with the disease characteristics of SLE patients is presented below:

Disease characteristics	SLEDAI- 2K	Rash (n /%)	Alopecia (n /%)	Arthritis (n /%)	Ulcers (n /%)	Fever (n /%)
SLE (n=14)	3 ± 4.3	5 / 35.7%	2 / 14.3%	8 / 57.1%	4 / 28.6%	2 / 14.3%

Disease characteristics	ANA positivity (n /%)	dsDNA positivity (n /%)	Low complement (n /%)	ESR (mean ± SD)
SLE (n=14)	12 / 85.7%	5 / 35.7%	9 / 64.3%	26.38 ± 18.96

Table 4: *Clinical and laboratory characteristics of the SLE subgroup.*

- A table with the disease characteristics of SSc patients is presented below:

Disease characteristics	Phenomenon Raynaud's (n /%)	Puffy fingers (n /%)	Digital ulcers (n /%)	Pulmonary fibrosis (n /%)	Telangiectasia (n /%)
SSc (N=9)	8 / 88.9%	7 / 77.8%	4 / 44.4%	3 / 33.3%	3 / 33.3%

Disease characteristics	ANA positivity (n /%)	ACA positivity (n /%)	Scl-70 positivity (n /%)	ESR (mean± SD)
SSc (N=9)	8 / 88.9%	1 / 11.1%	4 / 44.4%	28.38 ± 21.8

Table 5: *Clinical and laboratory characteristics of the SSc subgroup.*

▪ A table with the disease characteristics of ABD patients is presented below:

Disease characteristics	mouth sores (n / %)	genital ulcers (n / %)	skin rash (n /%)	arthritis (n / %)	fever (n / %)
ABD (N=6)	6 / 100.%	3 / 50.%	4 / 66.7%	2 / 33.3%	6 / 100.%

Disease characteristics	ocular involvement (n / %)	CNS involvement (n / %)	GI involvement (n / %)	ESR (mean ± SD)
APS (N=40)	2 / 33.3%	1 / 16.7%	0 / 0%	14.6 ± 12.58

Table 6: *Clinical and laboratory characteristics of the ABD subgroup.*

▪ A table with the disease characteristics of APS patients is presented below:

Disease characteristics	Primary APS (n / %)	SLE -APS (n / %)	Venous Thrombosis (n / %)	Arterial Thrombosis (n / %)
APS (N=40)	34 / 85.0%	6 / 15.0%	31 / 77.5%	14 / 35.0 %

Disease characteristics	Myocardial Infarction (n /%)	Stroke (n / %)	Transient ischemic attack n,%	Cerebral vascular accident n,%	Peripheral arterial disease n,%	Hypertension n,%
APS (N=40)	3 / 7.5 %	4 / 10.%	4 / 10.%	5 / 12.5%	1 / 2.5%	10 / 25.%

Disease characteristics	Dyslipidemia (n / %)	anti-La presence (n / %)	β2 GPI presence (n / %)	aCL presence (n/%)	CRP (mg/dl)
APS (N=40)	7 / 17.5%	31 / 77.5%	24 / 60.%	31 / 77.5%	2.7 ± 2.77

Table 7: *Clinical and laboratory characteristics of the APS subgroup.*

4.2 Increased levels of oxidative stress in patients with systemic autoimmune diseases.

Peripheral blood mononuclear cells (PBMCs) were isolated from patients and healthy individuals within 2h from venipuncture The presence of oxidative stress in PBMCs was evaluated by quantifying the total (GSH) and oxidized glutathione (GSSG) and calculating their ratio. Glutathione constitutes the most abundant free-thiol intracellular antioxidant and thus was selected as the distinctive molecule for measuring intracellular oxidative stress. In order to minimize the effect of sex and age, 1:3 age- and sex- matched healthy individuals were selected to be examined against each disease subgroup.

Endogenous basal oxidative stress was significantly elevated in the PBMCs of patients with systemic autoimmune diseases. Patients of each disease subgroup demonstrate significantly increased oxidative stress levels compared to the age- and sex- matched healthy group. As shown in Fig. 13., every disease subgroup exhibit comparable oxidative stress levels. In order of magnitude, ABD and APS patients displayed the highest levels of oxidative stress (**ABD**: 30.83 ± 8.33/ **HC**: 74.2 ± 8.54 [$p<0.001$] , **APS**: 44.86 ± 11.54 / **HC**: 69±11.33 [$p<0.001$]), followed by the RA, SLE and SSc patients (**RA**: 46.2 ± 8,56 / **HC**: 70±10.7 [$p<0.001$], **SLE**: 49.86 ± 12.6 / **HC**: 70 ±8.6 [$p<0.001$], **SSc**: 52 ± 15.68 / **HC**: 67.29 ± 10.1, [$p<0.01$]).

Figure 13.

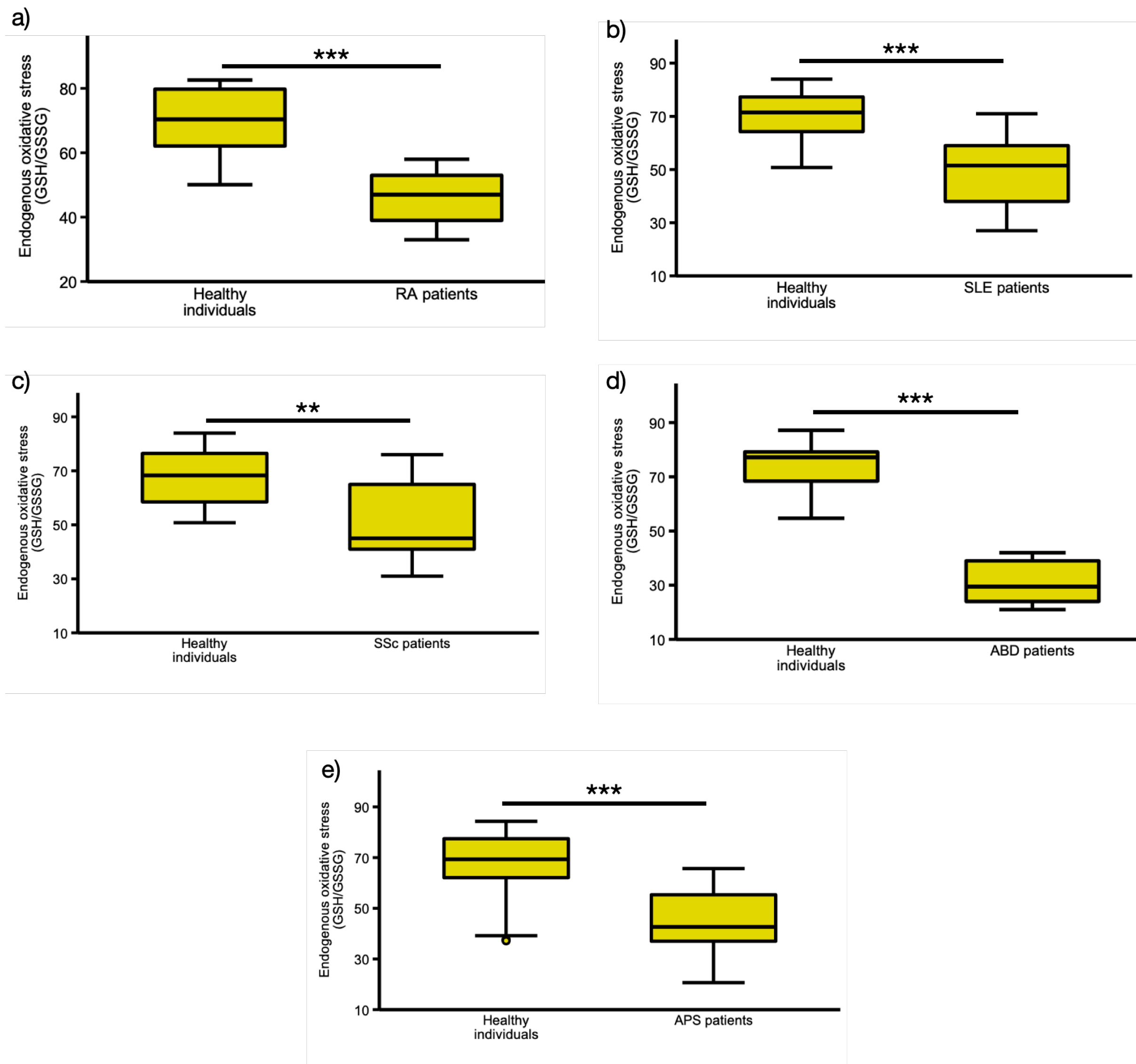


Figure 13: *Increased endogenous oxidative stress in patients with systemic autoimmune diseases (RA, SLE , SSc, ABD, APS), compared to 1:3 age- and sex- matched healthy individuals. The oxidative stress levels are comparable across every disease subtype. Tukey boxplots representing the basal oxidative stress levels, expressed as the ratio of reduced Glutathione (GSH) to oxidized glutathione (GSSG) in PBMCs derived from patients with systemic autoimmune diseases and 1:3 age- and sex- matched healthy controls. P-values are derived from Independent-Samples Mann-Whitney U Test. **p < 0.01, *** p<0.001.*

4.3 Increased abasic site formation in patients with systemic autoimmune diseases.

Subsequently, the endogenous formation of abasic sites was assessed with specified photometric method, as previously described, in the PBMCs of patients with systemic autoimmune diseases (RA, SLE , SSc, ABD, APS) and 1:3 age- and sex- matched healthy individuals. Abasic sites comprise one of the initial DNA damage forms after DNA oxidation. Thus, abasic site formation rate can be counted as a link between intracellular oxidative stress and DNA damage accumulation.

As shown in Figure 14, patients with systemic rheumatic diseases demonstrated elevated abasic site formation levels compared to the age- and sex- matched healthy group. Furthermore, this increase was found to be universal among the systemic rheumatic diseases examined in our study, further indicating that this oxidative stress induced damage is not a disease specific epiphenomenon (**RA**: 16.3 ± 3.2/ **HC**: 7.8±2.4 [p<0.001],], **SLE**: 14.8±4 / **HC**: 7±2.4 [p<0.001], **SSc**: 12.56 ± 3.2 / **HC**: 7 ±2.4 [p<0.01], **ABD**: 23 ± 8.12/ **HC**: 5.7 ± 1.4 [p<0.001], **APS**: 16.6 ± 6.7/ **HC**: 7.3 ± 3.04 [p<0.001]).

Figure 14.

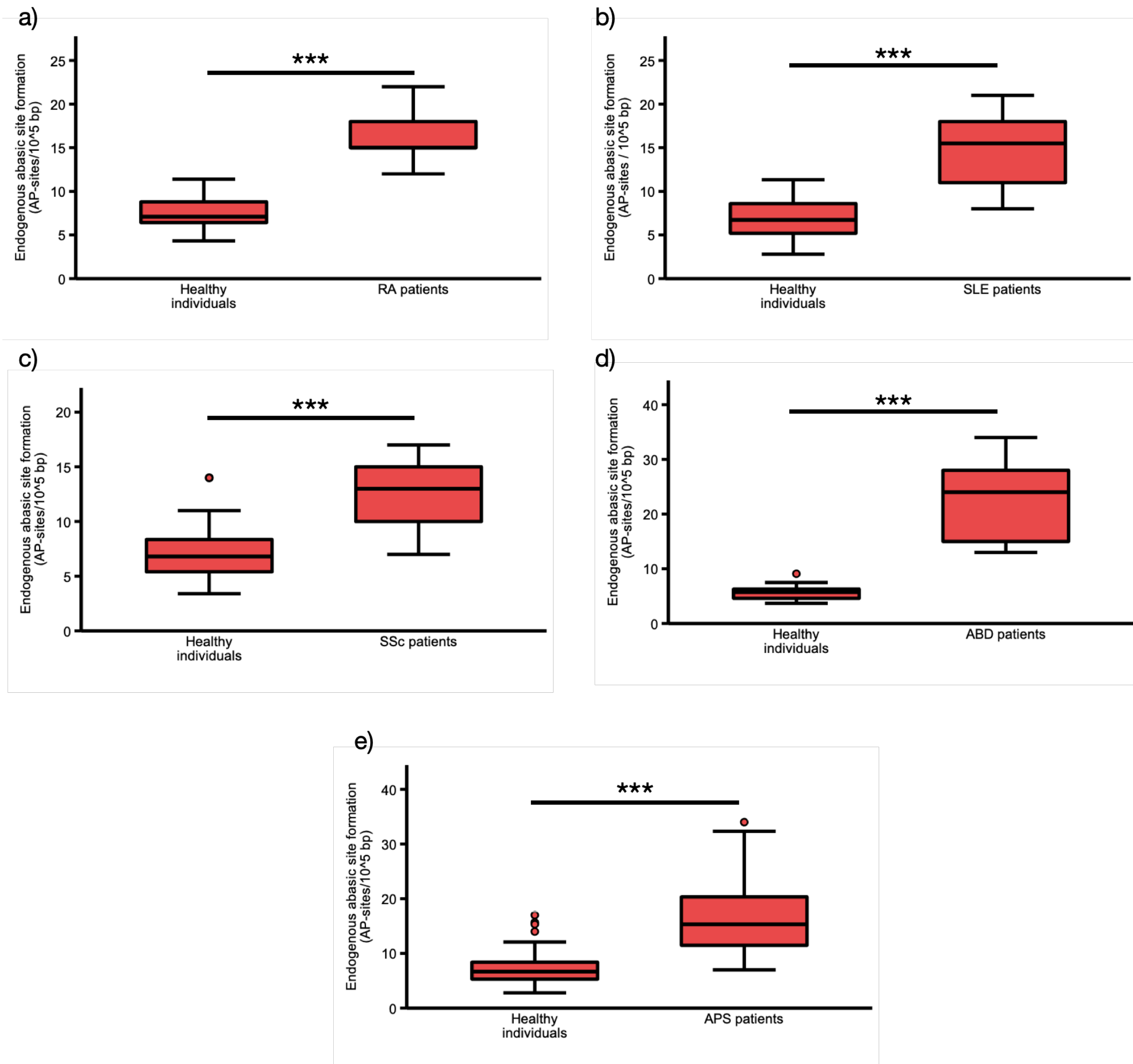


Figure 14**: Elevated abasic site formation levels in patients with systemic autoimmune diseases, compared to 1:3 age- and sex- matched healthy individuals. Tukey boxplots representing the abasic site formation levels, examining the endogenous AP-site formation, in PBMCs retrieved from patients with systemic autoimmune diseases and 1:3 age- and sex-matched healthy controls. P-values are derived from Independent-Samples Mann-Whitney U Test. $p<0.001$.*

4.3 The interplay of the intracellular oxidative stress and DNA damage formation

Next, to corroborate the association between intracellular oxidative stress and DNA damage formation levels, we examined whether the observed oxidative stress and the abasic site formation levels may be associated in our cohort. As it is already mentioned, oxidative stress, via ROS formation and accumulation can lead to glutathione oxidation and consequently to DNA damage formation, initially in the form of abasic sites.

As depicted in Figure 15, we found that both healthy individuals and patients with systemic autoimmune diseases demonstrate a strong correlation between oxidative stress and the endogenous abasic site formation levels (**HC**: $r=-0.555$, $p<0.001$, **Patients**: $r=-0.418$, $p<0.001$), denoting that the link between oxidative stress and abasic site formation is universal among the healthy and patients' groups.

Figure 15.

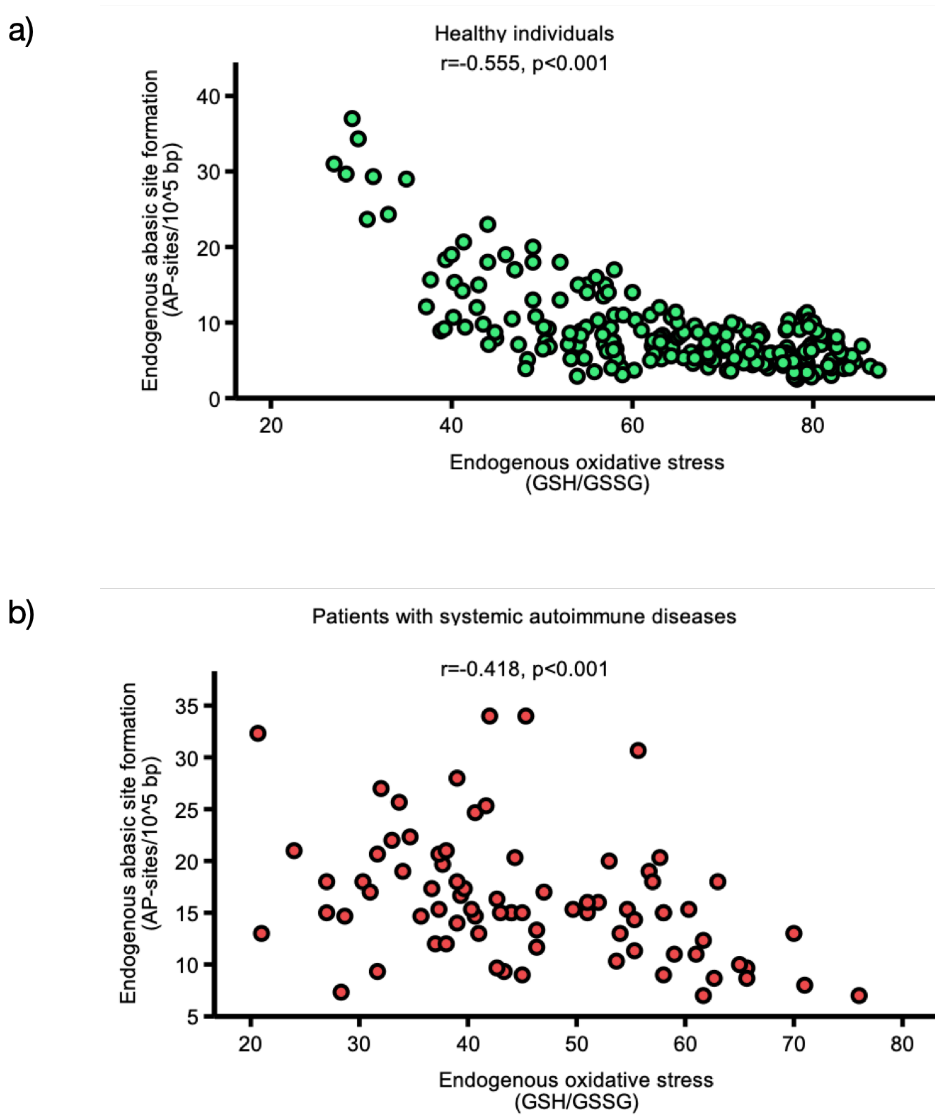


Figure 15*: Scatterplots depicting the association of endogenous oxidative stress (glutathione oxidation ratio / GSH to GSSG ratio) and abasic site formation levels in healthy individuals (N=212) and patients with systemic autoimmune diseases (N=78) . Correlation co-efficients are derived from Spearman's test.*

4.4 Lack of association between chronological age and oxidative stress in patients with systemic autoimmune diseases

Next, we aimed to examine whether chronological age can influence the intracellular oxidative stress status in both healthy individuals and patients with systemic autoimmune diseases and whether this association is differentiated between these groups. Searching the current bibliography, organismal aging has been associated with increased oxidative stress. Moreover, systemic autoimmune diseases can be demonstrated both in young and older age. Therefore, we sought to examine whether the above observed oxidative stress and oxidative DNA damage may be influenced by the chronological age.

In our study, we found that a strong association is present between oxidative stress and chronological age in the healthy individuals' group (Fig. 16. a) ($r=0.636$, $p<0.001$). However, in the patients' group this association is absent (Fig.16. c) ($r=0.208$, $p=0.067$), confirming a link between aberrant oxidative stress and systemic autoimmune diseases.

Furthermore, we examined also the association between abasic site formation rates and chronological age and whether they are also differentiated between healthy and patients' groups, since intracellular oxidative stress and abasic site formation are strongly associated. As expected, it was found that abasic site formation associated with chronological age in the healthy group only (Fig. 16b) ($r=0.734$, $p<0.001$), further corroborating the link between aberrant oxidative stress and systemic autoimmune diseases.

Figure 16.

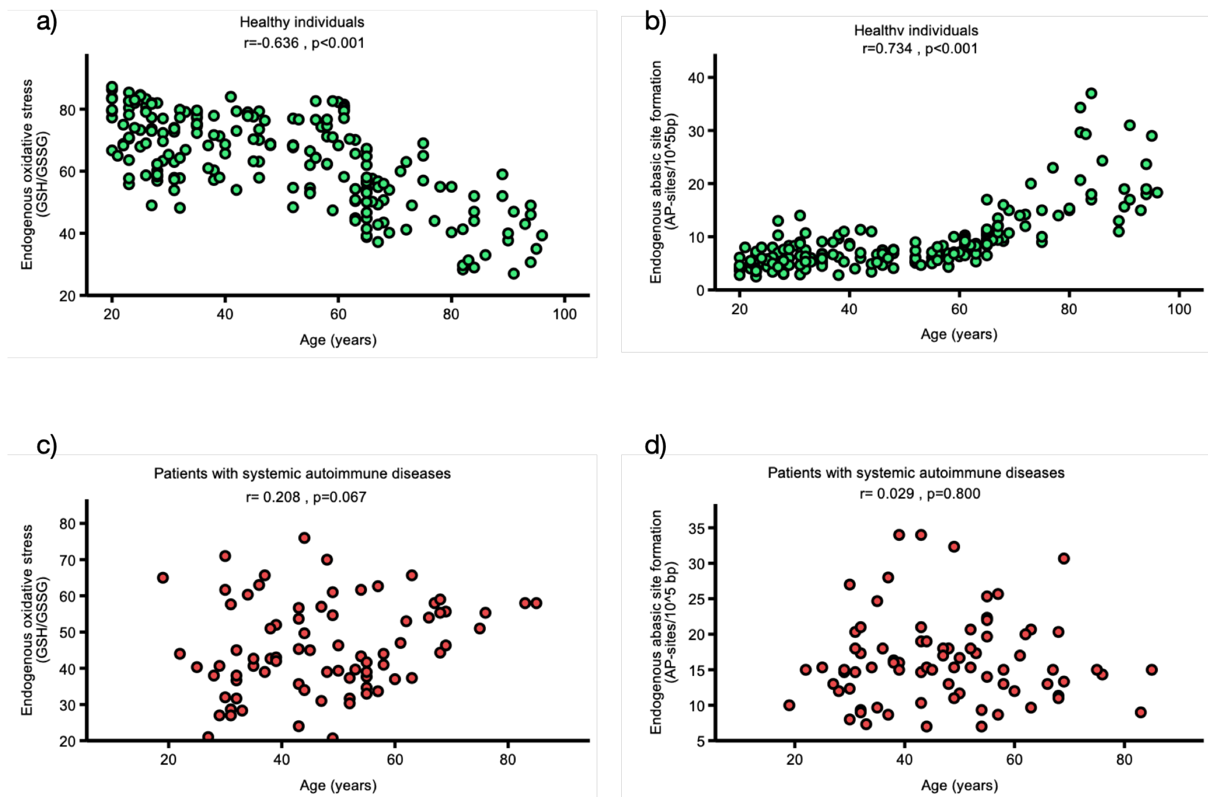


***Figure 16**: Scatterplots depicting the association of endogenous oxidative stress (glutathione oxidation ratio / GSH to GSSG ratio), abasic site formation levels and chronological age in both healthy individuals (N=212) and patients with systemic autoimmune diseases (N=78) . Correlation co-efficients are derived from Spearman's test.*

4.5 DNA damage accumulation in patients with systemic autoimmune diseases

In the next step, we studied the intracellular levels of the two most cytotoxic forms of DNA damage, the single-strand and double-strand DNA breaks (SSBs and DSBs). These lesions can be caused either by exogenous factors such as the action of ionizing radiation and UV-light, or as the final byproduct of intracellular DNA damaging agents, such as intracellular oxidative stress. In order to quantify these DNA lesions, we used the alkaline comet assay, which is capable of quantifying both these DNA lesions.

As depicted in Fig. 17, DNA damage accumulation is increased among every disease subgroup compared to 1:3 age- and sex- matched healthy individuals. It is of interest, that DNA damage accumulation is comparable across every disease subtype, confirming that this aberration is not disease-specific but a distinctive characteristic of systemic autoimmunity. In particular, RA- and APS- subgroups exhibited the highest DNA damage levels (Fig. 17 a, e) (**RA**: 15.7±8.5 / **HC**: 6.2±1.7 [p<0.001], **APS**: 14.5 ± 7.4 / **HC**: 5.3 ± 2 [p<0.001]), followed by the SLE-, SSc- and ABD- subgroups (Fig. 18 b-d) (**SLE**: 9.7± 5.4 / **HC**: 4.7 ± 1.5 [p<0.001], **SSc**: 11.8 ± 8 / **HC**: 4.8± 1.8 [p<0.001], **ABD**: 9.4± 5.7 / **HC**: 4± 1.3 [p<0.01]).

Figure 17.

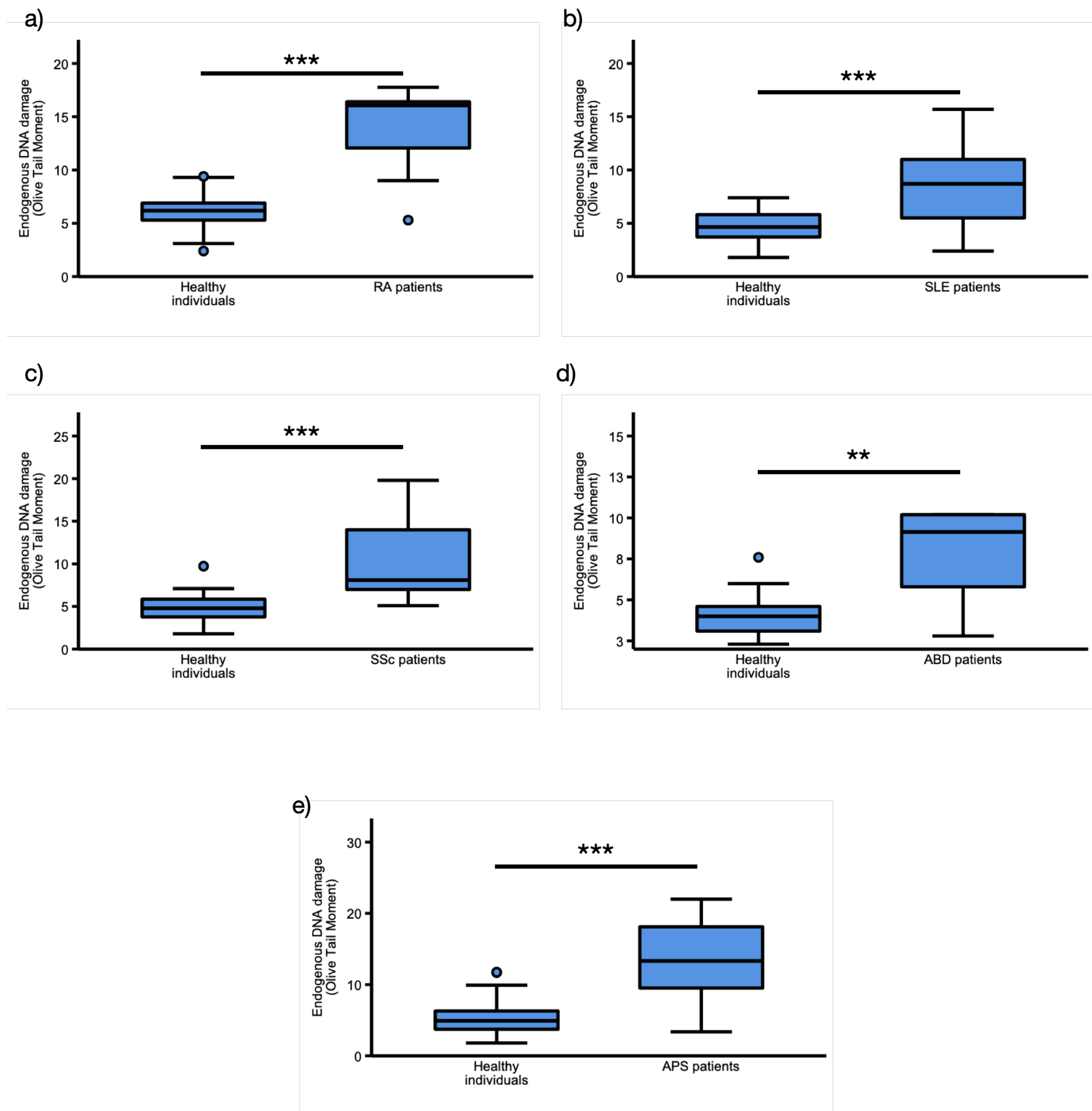


Figure 17. *Tukey boxplots representing the endogenous DNA damage levels, represented by the double-strand and single-strand DNA breaks, using the the single-cell gel electrophoresis under alkaline conditions (alkaline comet assay), in PBMCs derived from patients with systemic autoimmune diseases and 1:3 age- and sex- matched healthy controls. P-values are derived from Independent-Samples Mann-Whitney U Test. *** p<0.001.*

4.6 DNA damage accumulation significantly correlates with intracellular oxidative stress in patients with systemic autoimmune diseases

Next, we aimed to study, whether this increased DNA damage accumulation in the PBMCs of the patients with systemic autoimmune diseases can be associated with the corresponding aberrant oxidative stress, observed also in these patients. Thus we examined the possible correlations between DNA damage (both SSBs and DSBs), assessed by the alkaline comet assay, and the glutathione oxidation ratio (GSH / GSSG) or the abasic site formation levels (AP-sites).

Of interest, we found that endogenous DNA damage levels strongly correlated with both glutathione oxidation (Fig. 18a) ($r=-0.388$, $p<0.001$) and AP-site levels (Fig. 18b) ($r=0.455$, $p<0.001$), underlining a possible oxidative source for the accumulation of the double-strand and single-strand DNA breaks in patients with systemic autoimmune diseases.

Figure 18.

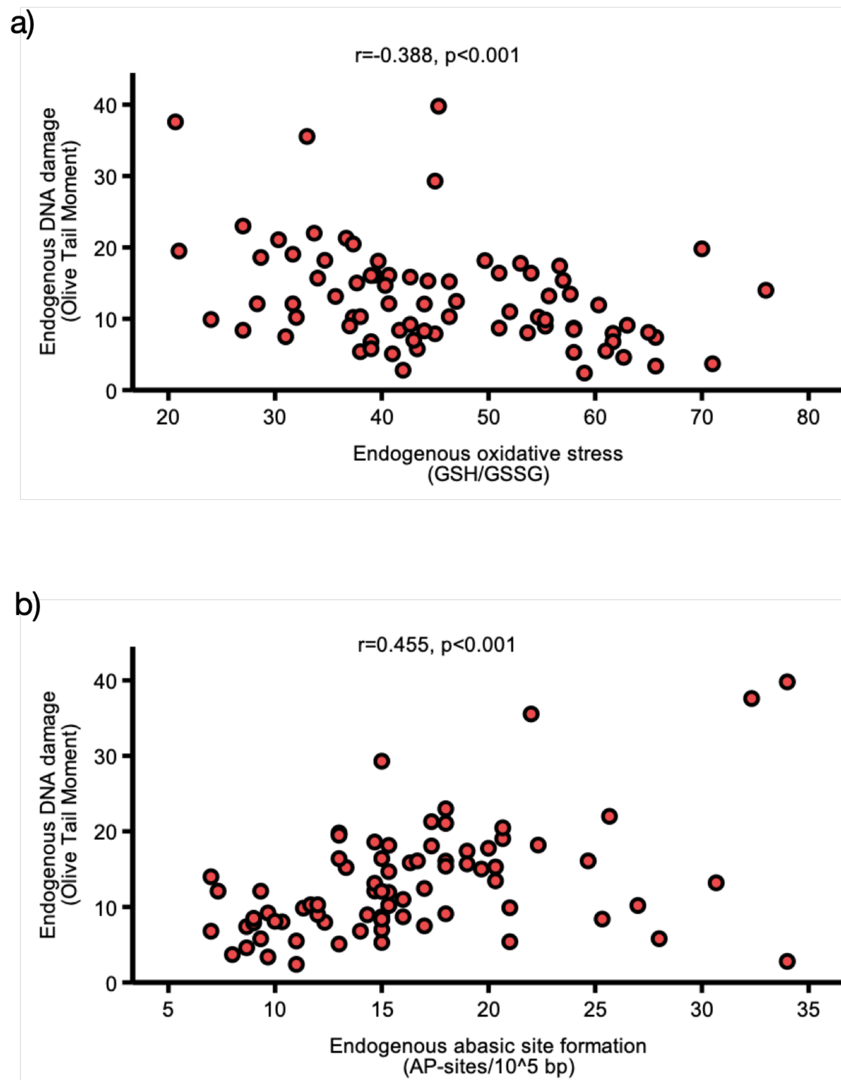


Figure 18: *Scatterplots depicting the association of endogenous oxidative stress (glutathione oxidation ratio / GSH to GSSG ratio), abasic site formation levels and endogenous DNA damage levels, represented by the double-strand and single-strand DNA breaks (OTM / arbitrary units) in both healthy individuals (N=212) and patients with systemic autoimmune diseases (N=78) . Correlation co-efficients are derived from Spearman's test.*

4.7 The interplay of intracellular oxidative stress and DNA damage repair capacity

Subsequently, we aimed to examine a possible relationship between the cellular DNA damage repair capacity and the intracellular oxidative stress. Since it was shown that intracellular oxidative stress, assessed by the glutathione oxidation ratio, strongly associated with both oxidative DNA damage (abasic site levels) and the following cytotoxic single-strand and double-strand DNA breaks in both healthy individuals and patients with systemic autoimmune diseases, we searched whether this intracellular oxidative status is also associated with the cellular DNA damage repair capacity.

Under physiological conditions, oxidative stress induces a transient formation of oxidative DNA damage, which is successfully repaired by the cellular DNA damage repair mechanisms. This oxidative DNA damage can initially be in the form of abasic sites and later due to the DNA repair mechanisms can take the form of double-strand and single-strand DNA breaks. Every form of DNA damage can be repaired by a specific DNA repair mechanism. In case of the double-strand DNA breaks (DSBs), they are repaired by double-strand DNA break repair (DSB-R) mechanism. The DSB-R was assessed by studying the γ-phosphorylation of the histone H2AX, one of the most sensitive DSB markers, using immunofluorescence antigen staining and confocal laser microscopy.

It was found that intracellular oxidative stress levels are strongly associated with double-strand break repair mechanism capacity in healthy individuals (Fig.19a) ($r=-0.500$, $p<0.001$). However, this association seems to be absent in patients with systemic autoimmune diseases, confirming the deficient DNA damage repair capacity, also shown in our previous studies (Fig.19b) ($r=-0.118$ $p=0.304$).

Figure 19.

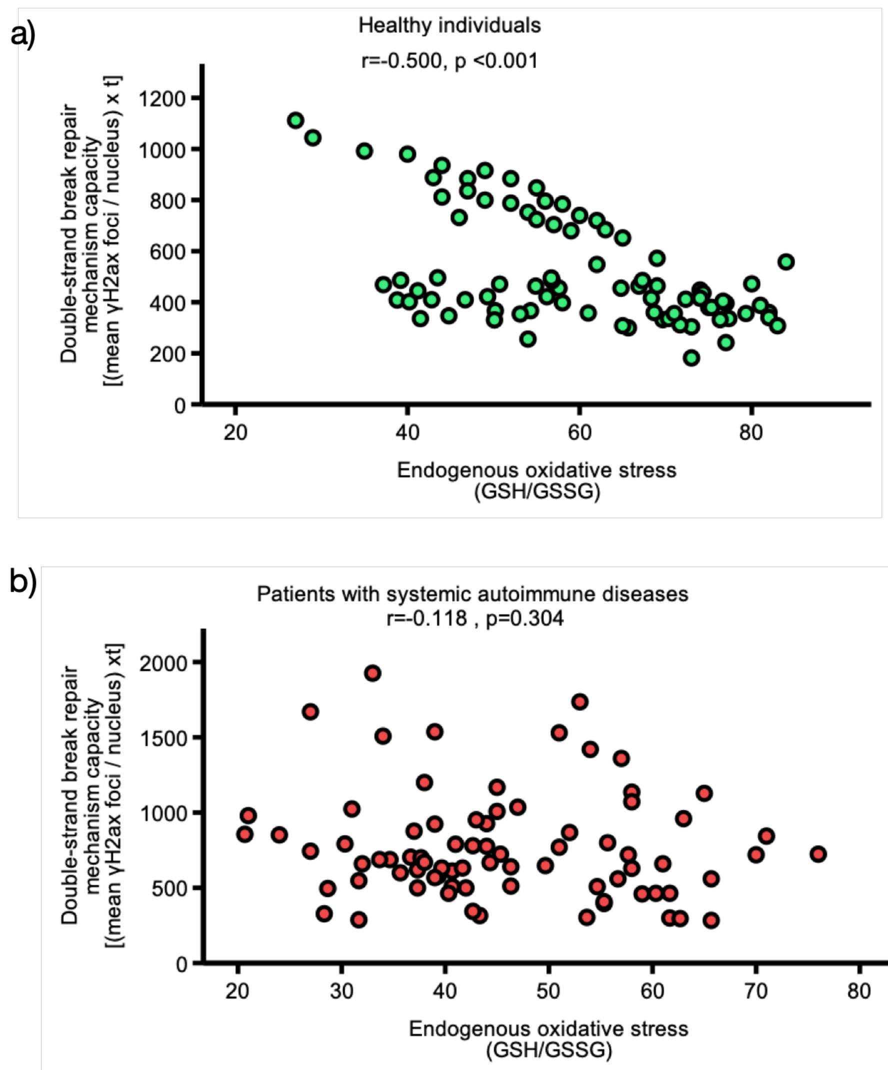


Figure 19: *Scatterplots demonstrating the association of endogenous oxidative stress (glutathione oxidation ratio / GSH to GSSG ratio) and the DNA damage repair capacity, represented by the double-strand DNA break repair mechanism capacity in both healthy individuals (N=83) and patients with systemic autoimmune diseases (N=78) . Correlation co-efficients are derived from Spearman's test.*

4.8 Lack of association between DNA damage repair capacity and aging in patients with systemic autoimmune diseases

Moreover, we examined whether the double-strand break repair (DSB-R) capacity may be influenced by the individual's chronological age. Since, oxidative stress and oxidative DNA damage does not seem to influence repair capacity in patients with systemic autoimmune diseases, we wondered whether chronological age could affect the observed DNA damage repair capacity in these individuals.

Interestingly, chronological age appeared to strongly associate with DSB-R capacity only in the healthy individuals (Fig. 20a) ($r=0.781$, $p<0.001$), displaying reduced DNA damage repair capacity with increasing age. On the contrary, this lack of association in patients with systemic autoimmune diseases (Fig. 20b) ($r=0.032$, $p=0.781$) validates the link between systemic autoimmunity and defective DNA damage repair network.

Figure 20.

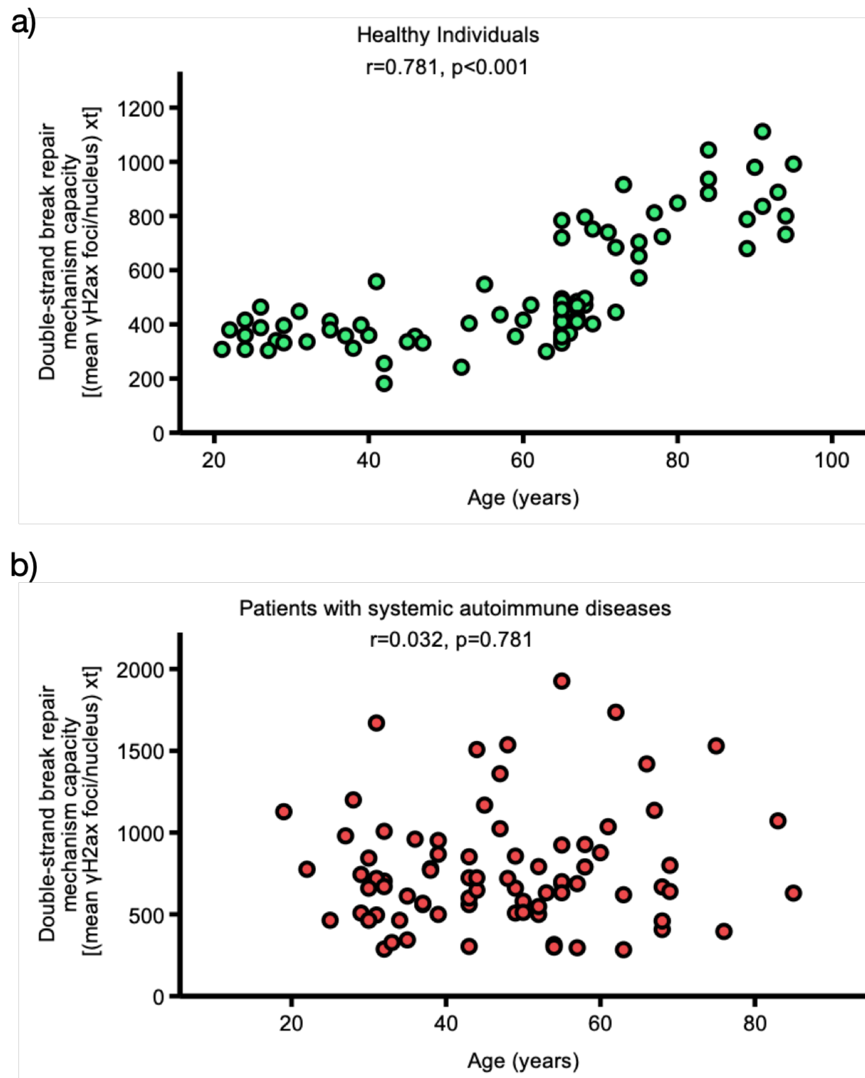


Figure 20: *Scatterplots showing the association of the DNA damage repair capacity, represented by the double-strand DNA break repair mechanism capacity and the chronological age in both healthy individuals (N=83) and patients with systemic autoimmune diseases (N=78) . Correlation co-efficients are derived from Spearman's test.*

4.9 Lack of association between endogenous DNA damage levels and aging in patients with systemic autoimmune diseases

Finally, to confirm the link between systemic autoimmunity and the defective DNA damage repair network, we examined the relationship between chronological age and endogenous DNA damage levels, expressed by both the single-strand and double-strand DNA breaks (DSBs and SSBs respectively), assessed by the alkaline comet assay in both healthy and patients' groups. Since neither oxidative stress and oxidative DNA damage, nor DSB-R capacity was influenced by chronological age in patients with systemic autoimmune diseases, we studied whether the intracellular levels of SSBs and DSBs were also thus affected.

Once again, chronological age was strongly associated with intracellular DNA damage levels only in the healthy group (Fig.21a) ($r=0.641$, $p<0.001$). In the group of the patients with systemic autoimmune diseases, chronological age again did not seem to influence the intracellular DNA damage levels, further corroborating the link between systemic autoimmune diseases and aberrant DNA damage repair capacity (Fig.21b) ($r=0.013$, $p=0.907$).

Figure 21.

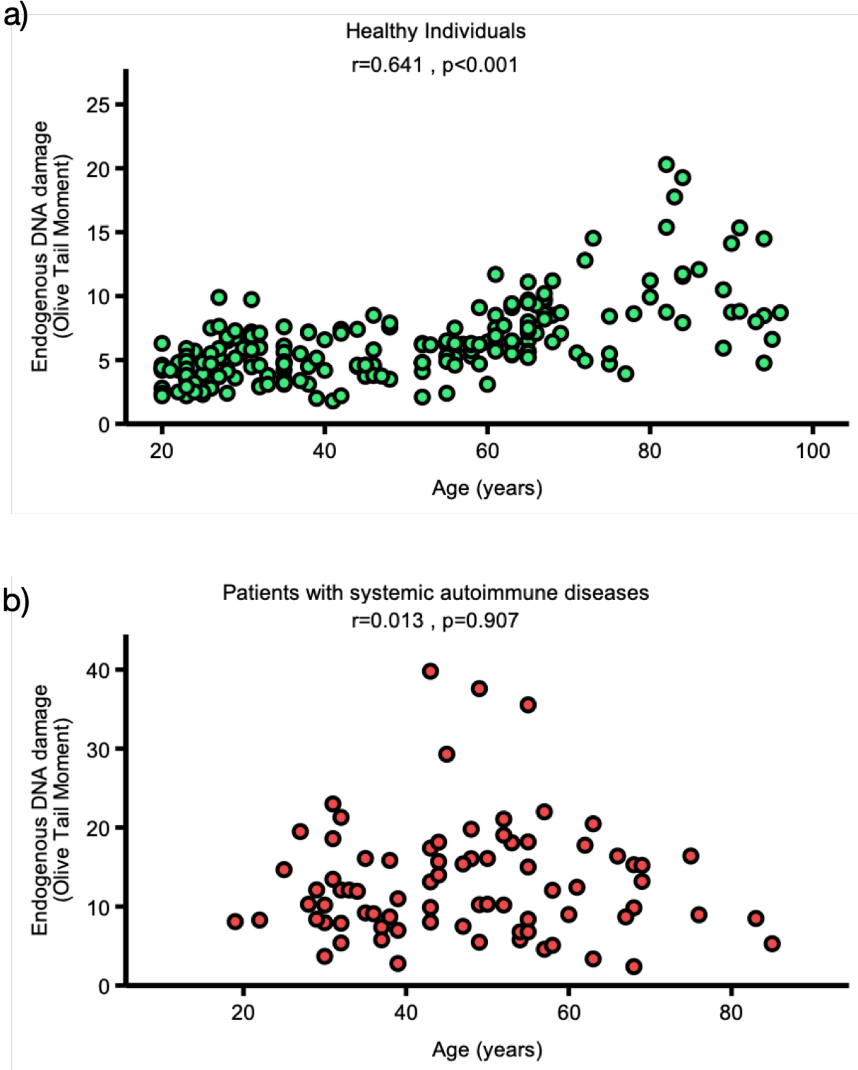


Figure 21: *Scatterplots showing the association of the DNA damage repair capacity, represented by the double-strand DNA break repair mechanism capacity and the chronological age in both healthy individuals (N=83) and patients with systemic autoimmune diseases (N=78) . Correlation co-efficients are derived from Spearman's test.*

5. Discussion

Herein, we present that increased accumulation of intracellular oxidative stress and DNA damage is evident in PBMCs of patients with systemic autoimmune diseases. Seventy eight (N=78) patients with systemic autoimmune diseases, including 9 patients with Rheumatoid Arthritis (RA), 14 patients with Systemic Lupus Erythematosus (SLE), 9 patients with Systemic Sclerosis (SSc), 6 patients with Adamantiades – Behcet's disease (ABD) and 40 patients with Antiphospholipid Syndrome (APS) were recruited. In order to assess intracellular oxidative stress, we studied the oxidization of GSH redox pair (GSH/GSSG), a key cellular antioxidant system. Furthermore, AP-site formation, the most frequent DNA lesions caused either spontaneously or by genotoxic insults, including oxidative stress, was also measured. Moreover, the two most cytotoxic forms of DNA damage, single-strand and double-strand DNA breaks, were quantified, using a single-cell gel electrophoresis assay (alkaline comet assay). Finally, the cellular double-strand DNA break repair (DSB-R) capacity, responsible for the removal of the cytotoxic DSBs, was quantified using immunofluorescence antigen staining and confocal laser microscopy.

First, increased oxidative stress and DNA damage accumulation were reported in PBMCs of patients with RA, compared to age- and sex- matched healthy individuals. This comes in line with previous results reporting increased levels of oxidative stress in correlation to endogenous DNA damage levels in neutrophiles and PBMCs of RA patients (182,183). Moreover, previous studies have shown increased levels of 8-oxodG in the DNA of peripheral blood lymphocytes, CD4+ T cells, and granulocytes of RA patients (64).

Next, as we have previously shown, SLE patients display augmented oxidative stress and DNA damage formation, quantified by both AP-site formation and single-strand and double-strand DNA breaks. These results come in line with data, reporting that neutrophils from SLE patients are characterized by increased accumulation of oxidative DNA damage and augmented apoptosis rates. This pro-oxidant status of the SLE neutrophiles could drive the enhanced generation of neutrophil extracellular traps (NETosis), observed in SLE patients, promoting the externalization of pro-inflammatory cytokines and further perpetuating the oxidative burden (184,185).

In case of Systemic Sclerosis, oxidative stress is theorized to be implicated in disease pathogenesis, although the exact mechanism remains yet unknown. Several studies have shown that fibroblasts retrieved from SSc patients exhibit increased oxidative stress, expressed by the increased ROS amount and decreased intracellular thiol levels, another significant intracellular antioxidant, compared to healthy fibroblasts (186,187). Moreover, patients with diffuse SSc and pulmonary fibrosis display increased levels of advanced oxidation protein products (AOPP) in their sera. AOPPS can drive the hydrogen peroxide production by endothelial cells and the proliferation of fibroblasts (188).

In Adamantiades – Behcet's disease, previous studies come in line with our data, showing that ABD patients display a prooxidant intracellular environment. Many immune cellular types, including neutrophils, lymphocytes and monocytes, retrieved from ABD patients manifest augmented prooxidant and decreased anti-oxidant mediators, leading to intracellular oxidative stress (105,189). This oxidative stress is theorized to play a pathogenetic role, since ROS from ABD neutrophiles negatively correlate with plasmin-induced fibrin lysis, suggesting an association with the prothrombotic environment observed in ABD (150). In agreement with our results displaying increased DNA damage in ABD patients, data from previous studies demonstrate increased genomic instability in PBMCs of ABD patients, as shown by an increased number of micro-nuclei and sister chromatin exchange events (190).

Furthermore, in this study we present augmented intracellular oxidative stress with a concurrent increase in DNA damage accumulation in patients with antiphospholipid syndrome. Of note, since the disease duration of the examined patients greatly varies, it is unclear whether these aberrations may act as a potent disease trigger or whether it is the result of the chronic immune activation. The oxidative stress involvement in APS can also be studied in previous reports, promoting several mechanisms of ROS production. For example, increased superoxide production was displayed possibly due to the circulation of aPL antibodies, resulting in increased plasma peroxynitrite levels, a highly pro-oxidant substance. On the contrary, anti-oxidant factors (paraoxonase-1 [PON1] activity, nitric oxide [NO] levels) and the overall intracellular anti-oxidant capacity has also been shown to be reduced, suggesting a permanent pro-oxidant environment, possibly leading to downregulation of inducible nitric oxide synthase expression and subsequent endothelial dysfunction (191,192).

Finally, we have shown that chronological age can affect the oxidative stress formation and DNA damage accumulation in healthy individuals, in contrast to the patients with systemic autoimmune diseases, where age does not seem to contribute. Of interest, it is displayed that in the case of the patients with autoimmune diseases individuals of young age can display comparable levels of oxidative stress and DNA damage to healthy individuals of old age and manifold higher than healthy individuals of the respective age. The effect of oxidative stress in the progression of the aging phenotype, which include four primary hallmarks (genomic instability, telomere attrition, epigenetic alterations, and loss of proteostasis), three antagonistic hallmarks (deregulated nutrient sensing, mitochondrial dysfunction and cellular senescence) and two integrative hallmarks (stem cell exhaustion and altered intercellular communication) has been previously studied with controversial results. These hallmarks, contributing to the ageing process, could be generated by oxidative damage due to the increased oxidative stress (193,194).

It is generally accepted that cellular health is endangered by increased DNA damage levels, since it can result in mutations and genomic instability. Several chronic clinical conditions, associated with aging, such as coronary artery disease, kidney disease, chronic obstructive pulmonary disease, multiple sclerosis, and Alzheimer's disease have demonstrated high DNA damage levels.(195,196) Furthermore, it is reported that increased DNA damage levels (quantified by the comet assay) may also act as a predictor of mortality risk, since it may represent a crucial factor in the development of chronic diseases and death (197). However, previous studies examining the effect of age on the accumulation of DNA damage in PBMCs of healthy individuals have reported little or no effect. A meta-analysis of 105 studies including 13,553 subjects, with the majority of study subjects having various comorbidities, displayed only a slight change of endogenous DNA damage with increasing age (198). Similarly, another study displayed a modest but significant association of DNA damage levels and chronological age in individuals aged between 40 and 77 years old (199).

6. Conclusion

In conclusion, our study demonstrates that patients with systemic autoimmune diseases exhibit increased intracellular oxidative stress, that is associated with increased DNA damage formation, both in the form of initial oxidative DNA damage (abasic site formation) and cytotoxic double-strand and single strand DNA breaks. Furthermore, DNA damage repair mechanisms in patients with systemic autoimmune diseases appear to be defective, suggesting that both increased DNA damage formation and aberrant DNA damage may influence the pathogenesis of systemic autoimmunity.

Moreover, we show that chronological age strongly correlates with increased oxidative and DNA damage burden and diminished DDR capacity in apparently healthy individuals. In contrast, age does not seem to affect either the cellular oxidative status or the DDR capacity in patients with systemic autoimmunity, pointing to the pathogenetic link between systemic autoimmunity and the aberrant DNA damage response.

Bibliography

1. Friedberg, E.C., 2008. A brief history of the DNA repair field. Cell Res 18, 3–7. https://doi.org/10.1038/cr.2007.113

2. Tubbs, A., Nussenzweig, A., 2017. Endogenous DNA Damage as a Source of Genomic Instability in Cancer. Cell 168, 644–656.

3. Jackson, S.P., Bartek, J., 2009. The DNA-damage response in human biology and disease. Nature 461, 1071–1078.

4. Mullenders, L.H.F., 2018. Solar UV damage to cellular DNA: from mechanisms to biological effects. Photochem Photobiol Sci 17, 1842–1852.

5. Wogan, G.N., Hecht, S.S., Felton, J.S., Conney, A.H., Loeb, L.A., 2004. Environmental and chemical carcinogenesis. Seminars in Cancer Biology 14, 473–486.

6. Ciccia, A., Elledge, S.J., 2010. The DNA damage response: making it safe to play with knives. Mol Cell 40, 179–204.

7. Chatterjee, N., Walker, G.C., 2017. Mechanisms of DNA damage, repair, and mutagenesis: DNA Damage and Repair. Environ. Mol. Mutagen. 58, 235–263.

8. Cooke, M.S., Evans, M.D., Dizdaroglu, M., Lunec, J., 2003. Oxidative DNA damage: mechanisms, mutation, and disease. FASEB j. 17, 1195–1214.

9. Souliotis, V.L., Vlachogiannis, N.I., Pappa, M., Argyriou, A., Ntouros, P.A., Sfikakis, P.P., 2019. DNA Damage Response and Oxidative Stress in Systemic Autoimmunity. Int J Mol Sci 21, 55.

10. Pateras, I.S., Havaki, S., Nikitopoulou, X., Vougas, K., Townsend, P.A., Panayiotidis, M.I., Georgakilas, A.G., Gorgoulis, V.G., 2015. The DNA damage response and immune signaling

alliance: Is it good or bad? Nature decides when and where. Pharmacology & Therapeutics 154, 36–56.

11. Lord, C.J., Ashworth, A., 2012. The DNA damage response and cancer therapy. Nature 481, 287–294.

12. Pilié, P.G., Tang, C., Mills, G.B., Yap, T.A., 2019. State-of-the-art strategies for targeting the DNA damage response in cancer. Nat Rev Clin Oncol 16, 81–104.

13. Rass, U., Ahel, I., West, S.C., 2007. Defective DNA Repair and Neurodegenerative Disease. Cell 130, 991–1004.

14. Kulkarni, A., Wilson, D.M., 2008. The Involvement of DNA-Damage and -Repair Defects in Neurological Dysfunction. The American Journal of Human Genetics 82, 539–566.

15. Amirifar, P., Ranjouri, M.R., Yazdani, R., Abolhassani, H., Aghamohammadi, A., 2019. Ataxia-telangiectasia: A review of clinical features and molecular pathology. Pediatr Allergy Immunol 30, 277–288.

16. Kitao, H., Takata, M., 2011. Fanconi anemia: a disorder defective in the DNA damage response. Int J Hematol 93, 417–424.

17. Manolakou, T., Verginis, P., Boumpas, D.T., 2021. DNA Damage Response in the Adaptive Arm of the Immune System: Implications for Autoimmunity. Int J Mol Sci 22, 5842.

18. Vl, S., Ni, V., M, P., A, A., Pp, S., 2019. DNA damage accumulation, defective chromatin organization and deficient DNA repair capacity in patients with rheumatoid arthritis. Clinical immunology (Orlando, Fla.) 203.

19. Souliotis, V.L., Vougas, K., Gorgoulis, V.G., Sfikakis, P.P., 2016. Defective DNA repair and chromatin organization in patients with quiescent systemic lupus erythematosus. Arthritis Res Ther 18, 182.

20. Vlachogiannis, N.I., Pappa, M., Ntouros, P.A., Nezos, A., Mavragani, C.P., Souliotis, V.L., Sfikakis, P.P., 2020. Association Between DNA Damage Response, Fibrosis and Type I Interferon Signature in Systemic Sclerosis. Front Immunol 11, 582401.

21. Zheng, X., Sawalha, A.H., 2022. The Role of Oxidative Stress in Epigenetic Changes Underlying Autoimmunity. Antioxid Redox Signal 36, 423–440.

22. Smallwood, M.J., Nissim, A., Knight, A.R., Whiteman, M., Haigh, R., Winyard, P.G., 2018. Oxidative stress in autoimmune rheumatic diseases. Free Radical Biology and Medicine 125, 3–14.

23. Gergely, P., Niland, B., Gonchoroff, N., Pullmann, R., Phillips, P.E., Perl, A., 2002. Persistent mitochondrial hyperpolarization, increased reactive oxygen intermediate production, and cytoplasmic alkalinization characterize altered IL-10 signaling in patients with systemic lupus erythematosus. J Immunol 169, 1092–1101.

24. Morgan, M.J., Liu, Z., 2011. Crosstalk of reactive oxygen species and NF-κB signaling. Cell Res 21, 103–115.

25. Perl, A., 2013. Oxidative stress in the pathology and treatment of systemic lupus erythematosus. Nat Rev Rheumatol 9, 674–686.

26. Dahlmann HA, Vaidyanathan VG, Sturla SJ. Investigating the biochemical impact of DNA damage with structure-based probes: abasic sites, photodimers, alkylation adducts, and oxidative lesions. Biochemistry. 2009 Oct 13;48(40):9347-59.

27. Kappen LS, Goldberg IH. Identification of 2-deoxyribonolactone at the site of neocarzinostatin-induced cytosine release in the sequence d(AGC) Biochemistry. 1989;28:1027–1032

28. Greenberg MM, Weledji YN, Kroeger KM, Kim J, Goodman MF. In vitro effects of a C4′-oxidized abasic site on DNA polymerases. Biochemistry. 2004;43:2656–2663

29. Zuo, Prather, Stetskiv, Garrison, Meade, Peace, Zhou, 2019. Inflammaging and Oxidative Stress in Human Diseases: From Molecular Mechanisms to Novel Treatments. IJMS 20, 4472.

30. Henle, E.S., Linn, S., 1997. Formation, Prevention, and Repair of DNA Damage by Iron/Hydrogen Peroxide *. Journal of Biological Chemistry 272, 19095–19098.

31. Friedberg, E.C., 2005. Suffering in silence: the tolerance of DNA damage. Nat Rev Mol Cell Biol 6, 943–953.

32. Malle, E., Furtmüller, P.G., Sattler, W., Obinger, C., 2007. Myeloperoxidase: a target for new drug development? British Journal of Pharmacology 152, 838–854.

33. Sies, H., 2015. Oxidative stress: a concept in redox biology and medicine. Redox Biol 4, 180–183.

34. Forman, H.J., Zhang, H., 2021. Targeting oxidative stress in disease: promise and limitations of antioxidant therapy. Nat Rev Drug Discov 20, 689–709.

35. Kudryavtseva, A.V., Krasnov, G.S., Dmitriev, A.A., Alekseev, B.Y., Kardymon, O.L., Sadritdinova, A.F., Fedorova, M.S., Pokrovsky, A.V., Melnikova, N.V., Kaprin, A.D., Moskalev, A.A., Snezhkina, A.V., 2016. Mitochondrial dysfunction and oxidative stress in aging and cancer. Oncotarget 7, 44879–44905.

36. Evans, M.D., Dizdaroglu, M., Cooke, M.S., 2004. Oxidative DNA damage and disease: induction, repair and significance. Mutation Research/Reviews in Mutation Research 567, 1 61

37. Sedelnikova, O.A., Redon, C.E., Dickey, J.S., Nakamura, A.J., Georgakilas, A.G., Bonner, W.M., 2010. Role of oxidatively induced DNA lesions in human pathogenesis. Mutat Res 704, 152–159.

38. Altieri, F., Grillo, C., Maceroni, M., Chichiarelli, S., 2008. DNA Damage and Repair: From Molecular Mechanisms to Health Implications. Antioxidants & Redox Signaling 10, 891–938.

39. Collins, A.R., Cadet, J., Mőller, L., Poulsen, H.E., Viña, J., 2004. Are we sure we know how to measure 8-oxo-7,8-dihydroguanine in DNA from human cells? Archives of Biochemistry and Biophysics, Oxygen Club of California: A Tribute to Bruce N. Ames 423, 57–65.

40. Dolinnaya, N.G., Kubareva, E.A., Romanova, E.A., Trikin, R.M., Oretskaya, T.S., 2013. Thymidine glycol: the effect on DNA molecular structure and enzymatic processing. Biochimie 95, 134–147.

41. Kryston, T.B., Georgiev, A.B., Pissis, P., Georgakilas, A.G., 2011. Role of oxidative stress and DNA damage in human carcinogenesis. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, From chemistry of DNA damage to repair and biological significance. Comprehending the future 711, 193–201.

42. Čolak, E., Ignjatović, S., Radosavljević, A., Žorić, L., 2017. The association of enzymatic and non-enzymatic antioxidant defense parameters with inflammatory markers in patients with exudative form of age-related macular degeneration. J Clin Biochem Nutr 60, 100–107.

43. Aoyama, K. Glutathione in the Brain. Int. J. Mol. Sci. 2021, 22, 5010.

44. Mari, M.; Morales, A.; Colell, A.; Garcia-Ruiz, C.; Kaplowitz, N.; Fernandez-Checa, J.C. Mitochondrial Glutathione: Features, Regulation and Role in Disease. Biochim. Biophys. Acta 2013, 1830, 3317–3328.

45. Wu, G.; Fang, Y.Z.; Yang, S.; Lupton, J.R.; Turner, N.D. Glutathione Metabolism and Its Implications for Health. J. Nutr. 2004, 134, 489–492.

46. Gilbert, H.F. Thiol/disulfide exchange equilibria and disulfide bond stability. Methods Enzym. 1995, 251, 8–28.

47. Enns, G.M.; Cowan, T.M. Glutathione as a Redox Biomarker in Mitochondrial Disease—Implications for Therapy. J. Clin. Med. 2017, 6, 50.

48. Moore, T.; Le, A.; Niemi, A.K.; Kwan, T.; Cusmano-Ozog, K.; Enns, G.M.; Cowan, T.M. A New Lc-Ms/Ms Method for the Clinical Determination of Reduced and Oxidized Glutathione from Whole Blood. J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 2013, 929, 51–55.

49. Giustarini, D.; Dalle-Donne, I.; Milzani, A.; Rossi, R. Detection of Glutathione in Whole Blood after Stabilization with N-Ethylmaleimide. Anal. Biochem. 2011, 415, 81–83.

50. Huang, W., Hickson, L.J., Eirin, A. et al. Cellular senescence: the good, the bad and the unknown. Nat Rev Nephrol 18, 611–627 (2022).

51. Gorgoulis, V.; Adams, P.D.; Alimonti, A.; Bennett, D.C.; Bischof, O.; Bishop, C.; Campisi, J.; Collado, M.; Evangelou, K.; Ferbeyre, G.; et al. Cellular Senescence: Defining a Path Forward. Cell 2019, 179, 813–827.

52. Rai, P.; Onder, T.T.; Young, J.J.; McFaline, J.L.; Pang, B.; Dedon, P.C.; Weinberg, R.A. Continuous elimination of oxidized nucleotides is necessary to prevent rapid onset of cellular senescence. Proc. Natl. Acad. Sci. USA 2009, 106, 169–174.

53. Lee, M.Y.; Wang, Y.; Vanhoutte, P.M. Senescence of cultured porcine coronary arterial endothelial cells is associated with accelerated oxidative stress and activation of NFkB. J. Vasc. Res. 2010, 47, 287–298.

54. Ito, K.; Hirao, A.; Arai, F.; Takubo, K.; Matsuoka, S.; Miyamoto, K.; Ohmura, M.; Naka, K.; Hosokawa, K.; Ikeda, Y.; et al. Reactive oxygen species act through p38 MAPK to limit the lifespan of hematopoietic stem cells. Nat. Med. 2006, 12, 446–451.

55. Li, G.; Luna, C.; Qiu, J.; Epstein, D.L.; Gonzalez, P. Alterations in microRNA expression in stress-induced cellular senescence. Mech. Ageing Dev. 2009, 130, 731–741.

56. Raje, N., Dinakar, C., 2015. Overview of Immunodeficiency Disorders. Immunology and Allergy Clinics of North America 35, 599–623.

57. Al-Herz W, Bousfiha A, Casanova JL, et al. Primary immunodeficiency diseases: an update on the classification from the international union of immunological societies expert committee for primary immunodeficiency. Front Immunol 2014;5: 162.

58. Theofilopoulos, A.N., Kono, D.H., Baccala, R., 2017. The multiple pathways to autoimmunity. Nat Immunol 18, 716–724.

59. Pan, P.-Y., Ozao, J., Zhou, Z., Chen, S.-H., 2008. Advancements in immune tolerance. Advanced Drug Delivery Reviews, Emerging Trends in Cell-Based Therapies 60, 91–105.

60. Kellie S, Al-Mansour Z. Overview of the Immune System. Micro- Nanotechnol Vaccine Dev. 2017;357:63–81.

61. Zheng X, Sawalha AH. The Role of Oxidative Stress in Epigenetic Changes Underlying Autoimmunity. Antioxid Redox Signal. 2022 Mar;36(7-9):423-440. doi: 10.1089/ars.2021.0066. Epub 2022 Jan 4. PMID: 34544258; PMCID: PMC8982122.

62. Souliotis, V.L.; Sfikakis, P.P. Increased DNA double-strand breaks and enhanced apoptosis in patients with lupus nephritis. Lupus 2015, 24, 804–815.

63. Palomino, G.M.; Bassi, C.L.; Wastowski, I.J.; Xavier, D.J.; Lucisano-Valim, Y.M.; Crispim, J.C.O.; Rassi, D.M.; Marques-Netom, J.F.; Sakamoto-Hojo, E.T.; Moreau, P.; et al. Patients with systemic sclerosis present increased DNA damage differentially associated with DNA repair gene polymorphisms. J. Rheumatol. 2014, 41, 458–465.

64. Souliotis, V.L.; Vlachogiannis, N.I.; Pappa, M.; Argyriou, A.; Sfikakis, P.P. DNA damage accumulation, defective chromatin organization and deficient DNA repair capacity in patients with rheumatoid arthritis. Clin. Immunol. 2019, 203, 28–36.

65. Tektonidou MG, Lewandowski LB, Hu J, Dasgupta A, Ward MM. Survival in adults and children with systemic lupus erythematosus: a systematic review and Bayesian meta-analysis of studies from 1950 to 2016. Ann Rheum Dis 2017; 76: 2009–16.

66. Dörner, T., Furie, R., 2019. Novel paradigms in systemic lupus erythematosus. The Lancet 393, 2344–2358.

67. Hochberg MC. Systemic lupus erythematosus. Rheum Dis Clin North Am. 1990;16(3):617–39.

68. Sullivan, K. E. Genetics of systemic lupus erythematosus: clinical implications. Rheum. Dis. Clin. North Am. 26, 1229–1256 (2000).

69. Kaul, A., Gordon, C., Crow, M.K., Touma, Z., Urowitz, M.B., Van Vollenhoven, R., Ruiz-Irastorza, G., Hughes, G., 2016. Systemic lupus erythematosus. Nat Rev Dis Primers 2, 16039.

70. James, J. A. Clinical perspectives on lupus genetics: advances and opportunities. Rheum. Dis. Clin. North Am. 40, 413–432 (2014).

71. Graham, R. R. et al. Specific combinations of HLA-DR2 and DR3 class II haplotypes contribute graded risk for disease susceptibility and autoantibodies in human SLE. Eur. J. Hum. Genet. 15, 823–830 (2007).

72. Niewold, T. B. et al. Association of the IRF5 risk haplotype with high serum interferon-α activity in systemic lupus erythematosus patients. Arthritis Rheum. 58, 2481–2487 (2008).

73. Du, J. et al. DNA methylation pathways and their crosstalk with histone methylation. Nat. Rev. Mol. Cell Biol. 16, 5519–5532 (2015).

74. Kang, I. et al. Defective control of latent Epstein–Barr virus infection in systemic lupus erythematosus. J. Immunol. 172, 1287–1294 (2004).

75. Kirou, K. A. et al. Activation of the interferon-α pathway identifies a subgroup of systemic lupus erythematosus patients with distinct serologic features and active disease. Arthritis Rheum. 52, 1491–1503 (2005).

76. Gao, D. et al. Activation of cyclic GMP–AMP synthase by self-DNA causes autoimmune diseases. Proc. Natl Acad. Sci. USA 11 2 , E5699–E5705 (2015).

77. Oliveira, L. et al. Dysregulation of antiviral helicase pathways in systemic lupus erythematosus. Front. Genet. 5, 418 (2014).

78. Perl, A., 2013. Oxidative stress in the pathology and treatment of systemic lupus erythematosus. Nat Rev Rheumatol 9, 674–686.

79. Nagy, G., Koncz, A. & Perl, A. T cell activation- induced mitochondrial hyperpolarization is mediated by Ca2+- and redox-dependent production of nitric oxide. J. Immunol. 171, 5188–5197 (2003).

80. Caza, T. N. et al. HRES-1/RAB4-mediated depletion of DRP1 impairs mitochondrial homeostasis and represents a target for treatment in SLE. Ann. Rheum. Dis. http:// ard.bmj.com/content/early/2013/07/29/ annrheumdis-2013-203794.

81. Gergely, P. J. et al. Mitochondrial hyperpolarization and ATP depletion in patients with systemic lupus erythematosus. Arthritis Rheum. 46, 175–190 (2002).

82. Gergely, P. J. et al. Persistent mitochondrial hyperpolarization, increased reactive oxygen intermediate production, and cytoplasmic alkalinization characterize altered IL-10 signaling in patients with systemic lupus erythematosus. J. Immunol. 169, 1092–1101 (2002).

83. Shah, D., Aggarwal, A., Bhatnagar, A., Kiran, R. & Wanchu, A. Association between T lymphocyte sub-sets apoptosis and peripheral blood mononuclear cells oxidative stress in systemic lupus erythematosus. Free Rad. Res. 45, 559–567 (2011).

84. Bedard, K. & Krause, K. H. The NOX family of ROS-generating NADPH oxidases: Physiology and pathophysiology. Physiol. Rev. 87, 245–313 (2007).

85. Segal, B. H., Grimm, M. J., Khan, A. N., Han, W. & Blackwell, T. S. Regulation of innate immunity by NADPH oxidase. Free Rad. Biol. Med. 53, 72–80 (2012).

86. Schärer OD. Nucleotide excision repair in Eukaryotes. Cold Spring Harbor Perspectives in Biology. 2013.

87. Smith JA, Bannister LA, Bhattacharjee V, Wang Y, Waldman BC, Waldman AS. Accurate Homologous Recombination Is a Prominent Double-Strand Break Repair Pathway in Mammalian Chromosomes and Is Modulated by Mismatch Repair Protein Msh2. Mol Cell Biol. 2007

88. Smolen, J.S., Aletaha, D., McInnes, I.B., 2016. Rheumatoid arthritis. Lancet 388, 2023–2038.

89. Alamanos Y, Voulgari PV, Drosos AA. Incidence and prevalence of rheumatoid arthritis, based on the 1987 American College of Rheumatology criteria: a systematic review. Semin Arthritis Rheum 2006; 36(3):182-188.

90. Thomas K, Lazarini A, Kaltsonoudis E, Drosos A, Papalopoulos I, Sidiropoulos P, et al. Multicenter Cross-sectional Study of Patients with Rheumatoid Arthritis in Greece: Results from a cohort of 2.491 patients. Mediterr J Rheumatol. 2018;29(1):27–37.

91. Uhlig T, Hagen KB, Kvien TK. Current tobacco smoking, formal education, and the risk of rheumatoid arthritis. J Rheumatol 1999; 26(1):47-54.

92. Millar K, Lloyd SM, McLean JS, Batty GD, Burns H, Cavanagh J et al. Personality, socio-economic status and inflammation: cross-sectional, population-based study. PLoS One 2013; 8(3):e58256.

93. Raychaudhuri S, Sandor C, Stahl EA, Freudenberg J, Lee HS, Jia X et al. Five amino acids in three HLA proteins explain most of the association between MHC and seropositive rheumatoid arthritis. Nat Genet 2012; 44(3):291-296.

94. Liu Y, Aryee MJ, Padyukov L, Fallin MD, Hesselberg E, Runarsson A et al. Epigenomewide association data implicate DNA methylation as an intermediary of genetic risk in rheumatoid arthritis. Nat Biotechnol 2013; 31(2):142-147.

95. Scher JU, Ubeda C, Equinda M, Khanin R, Buischi Y, Viale A et al. Periodontal disease and the oral microbiota in new-onset rheumatoid arthritis. Arthritis Rheum 2012; 64(10):3083-3094.

96. Wegner N, Wait R, Sroka A, Eick S, Nguyen KA, Lundberg K et al. Peptidylarginine deiminase from Porphyromonas gingivalis citrullinates human fibrinogen and alphaenolase: implications for autoimmunity in rheumatoid arthritis. Arthritis Rheum 2010; 62(9):2662-2672.

97. Scher JU, Littman DR, Abramson SB. Review: Microbiome in Inflammatory Arthritis and Human Rheumatic Diseases. Arthritis Rheumatol 2016; 68(1):35-45.

98. Scher JU, Sczesnak A, Longman RS, Segata N, Ubeda C, Bielski C et al. Expansion of intestinal Prevotella copri correlates with enhanced susceptibility to arthritis. Elife 2013; 2:e01202.

99. Anquetil F, Clavel C, Offer G, Serre G, Sebbag M. IgM and IgA Rheumatoid Factors Purified from Rheumatoid Arthritis Sera Boost the Fc Receptor– and Complement-Dependent Effector Functions of the Disease-Specific Anti–Citrullinated Protein Autoantibodies. J Immunol. 2015;

100. Firestein, G.S., McInnes, I.B., 2017. Immunopathogenesis of Rheumatoid Arthritis. Immunity 46, 183–196.

101. Scally, S.W., Petersen, J., Law, S.C., Dudek, N.L., Nel, H.J., Loh, K.L., Wijeyewickrema, L.C., Eckle, S.B., van Heemst, J., Pike, R.N., et al. (2013). A molecular basis for the association of the HLA-DRB1 locus, citrullination, and rheumatoid arthritis. J. Exp. Med. 210, 2569–2582.

102. Tan, E.M., and Smolen, J.S. (2016). Historical observations contributing insights on etiopathogenesis of rheumatoid arthritis and role of rheumatoid factor. J. Exp. Med. 213, 1937–1950.

103. da Fonseca, L.J.S., Nunes-Souza, V., Goulart, M.O.F., Rabelo, L.A., 2019. Oxidative Stress in Rheumatoid Arthritis: What the Future Might Hold regarding Novel Biomarkers and Add-On Therapies. Oxid Med Cell Longev 2019, 7536805.

104. Shao,L.; Fujii,H.; Colmegna,I.; Oishi,H.; Goronzy,J.J.; Weyand,C.M. Deficiency of the DNA repair enzyme ATM in rheumatoid arthritis. J. Exp. Med. 2009, 206, 1435–1449.

105. Bashir,S.; Harris,G.; Denman,M.A.; Blake,D.R.; Winyard,P.G. Oxidative DNA damage and cellular sensitivity to oxidative stress in human autoimmune diseases. Ann. Rheum. Dis. 1993, 52, 659–666.

106. Gabrielli A, Avvedimento EV, Krieg T. Scleroderma. N Engl J Med. 2009; 360(19):1989–2003.

107. Denton, C.P., Khanna, D., 2017. Systemic sclerosis. The Lancet 390, 1685–1699.

108. Tyndall AJ, Bannert B, Vonk M, et al. Causes and risk factors for death in systemic sclerosis: a study from the EULAR Scleroderma Trials and Research (EUSTAR) database. Ann Rheum Dis 2010; 69: 1809–15.

109. Pearson, D.R., Werth, V.P., Pappas-Taffer, L., 2018. Systemic sclerosis: Current concepts of skin and systemic manifestations. Clinics in Dermatology, Rheumatologic Dermatology 36, 459–474.

110. Wirz EG, Jaeger VK, Allanore Y, et al, and the EUSTAR co-authors. Incidence and predictors of cutaneous manifestations during the early course of systemic sclerosis: a 10-year longitudinal study from the EUSTAR database. Ann Rheum Dis 2016; 75: 1285–92.

111. Nihtyanova SI, Denton CP. Autoantibodies as predictive tools in systemic sclerosis. Nat Rev Rheumatol 2010; 6: 112–16.

112. van den Hoogen F, Khanna D, Fransen J, et al. 2013 classification criteria for systemic sclerosis: an American College of Rheumatology/European League against Rheumatism collaborative initiative. Arthritis Rheum 2013; 65: 2737–47.

113. Jordan S, Maurer B, Toniolo M, Michel B, Distler O. Performance of the new ACR/EULAR classification criteria for systemic sclerosis in clinical practice. Rheumatology 2015; 54: 1454–58.

114. Tyndall, A., Ladner, U.M., Matucci-Cerinic, M., 2008. The EULAR Scleroderma Trials and Research Group (EUSTAR): an international framework for accelerating scleroderma research. Curr Opin Rheumatol 20, 703–706.

115. Soldano S, Montagna P, Brizzolara R, et al. Effects of estrogens on extracellular matrix synthesis in cultures of human normal and scleroderma skin fibroblasts. Ann N Y Acad Sci. 2010;1193:25-29.

116. Arnett FC, Cho M, Chatterjee S, et al. Familial occurrence frequencies and relative risks for systemic sclerosis (scleroderma) in three United States cohorts. Arthritis Rheum 2001; 44(6): 1359–1362.

117. Gorlova O, Martin JE, Rueda B, et al. Identification of novel genetic markers associated with clinical phenotypes of systemic sclerosis through a genome-wide association strategy. PLoS Genet 2011; 7(7): e1002178.

118. Arnett FC, Gourh P, Shete S, et al. Major histocompatibility complex (MHC) class II alleles, haplotypes and epitopes which confer susceptibility or protection in systemic sclerosis: analyses in 1300 Caucasian, African-American and Hispanic cases and 1000 controls. Ann Rheum Dis 2010; 69(5): 822–827.

119. Mayes MD, Bossini-Castillo L, Gorlova O, et al. Immunochip analysis identifies multiple susceptibility loci for systemic sclerosis. Am J Hum Genet 2014; 94(1): 47–61.

120. Mora, G.F., 2009. Systemic sclerosis: environmental factors. J Rheumatol 36, 2383–2396.

121. Ward AM, Udnoon S, Watkins J, Walker AE, Darke CS. Immunological mechanisms in the pathogenesis of vinyl chloride disease. BMJ 1976;1:936–8.

122. Sanchez-Roman J, Wichmann I, Salaberri J, Varela JM, Nunez-Roldan A. Multiple clinical and biological autoimmune manifestations in 50 workers after occupational exposure to silica. Ann Rheum Dis 1993;52:534–8.

123. Yamakage A, Ishikawa H, Saito Y, Hattori A. Occupational scleroderma-like disorder occurring in men engaged in the polymerisation of epoxy resins. Dermatologica 1980;161:44.

124. Rosendahl, A.-H., Schönborn, K., Krieg, T., 2022. Pathophysiology of systemic sclerosis (scleroderma). The Kaohsiung Journal of Medical Sciences 38, 187–195.

125. Toledo DM, Pioli PA. Macrophages in systemic sclerosis: novel insights and therapeutic implications. Curr Rheumatol Rep. 2019; 21(7):31.

126. Roumm AD, Whiteside TL, Medsger TA Jr, Rodnan GP. Lymphocytes in the skin of patients with progressive systemic sclerosis. Quantification, subtyping, and clinical correlations. Arthritis Rheum. 1984; 27(6):645–53.

127. Denton CP, Abraham DJ. Transforming growth factor-beta and connective tissue growth factor: key cytokines in scleroderma pathogenesis. Curr Opin Rheumatol. 2001;13(6):505–11.

128. Hasegawa M, Fujimoto M, Kikuchi K, Takehara K. Elevated serum levels of interleukin 4 (IL-4), IL-10, and IL-13 in patients with systemic sclerosis. J Rheumatol. 1997;24(2):328–32.

129. Zurawski G, de Vries JE. Interleukin 13, an interleukin 4-like cytokine that acts on monocytes and B cells, but not on T cells. Immunol Today. 1994;15(1):19–26.

130. Vona, R., Giovannetti, A., Gambardella, L., Malorni, W., Pietraforte, D., Straface, E., 2018. Oxidative stress in the pathogenesis of systemic scleroderma: An overview. J Cell Mol Med 22, 3308–3314.

131. Sfrent-Cornateanu R, Mihai C, Stoian I, Lixandru D, Bara C, Moldoveanu E. Antioxidant defense capacity in scleroderma patients. Clin Chem Lab Med. 2008;46:836-841.

132. Kadono T, Kikuchi K, Ihn H, Takehara K, Tamaki K. Increased production of interleukin 6 and interleukin 8 in scleroderma fibroblasts. J Rheumatol. 1998;25:296-301.

133. Hatemi G, Silman A, Bang D, Bodaghi B, Chamberlain A, Gul A, Houman M, K€ otter I, Olivieri I, Salvarani C. 2008. EULAR recommendations for the management of Behc ̧et disease. Ann Rheumatic Dis. 67:1656–1662.

134. Cho S, Cho S, Bang D. 2012. New insights in the clinical understanding of Behcet's disease. Yonsei Med J. 53:35–42.

135. Salmaninejad, A., Gowhari, A., Hosseini, S., Aslani, S., Yousefi, M., Bahrami, T., Ebrahimi, M., Nesaei, A., Zal, M., 2017. Genetics and immunodysfunction underlying Behçet's disease and immunomodulant treatment approaches. Journal of Immunotoxicology 14, 137–151.

136. Takeuchi M, Kastner D, Remmers E. 2015. The immunogenetics of Behc ̧et's disease: A comprehensive review. J Autoimmun. 64:137–148.

137. Pineton de Chambrun M, Wechsler B, Geri G, Cacoub P, Saadoun D. 2012. New insights into the pathogenesis of Behc ̧et's disease. Autoimmun Rev. 11:687–698.

138. M. Ognenovski, P. Renauer, E. Gensterblum, I. K¨ otter, T. Xenitidis, J.C. Henes, B. Casali, C. Salvarani, H. Direskeneli, K.M. Kaufman, A.H. Sawalha, Whole exome sequencing identifies rare protein-coding variants in Behçet's disease, Arthritis Rheum. 68 (2016) 1272–1280.

139. Studd M, McCance D, Lehner T. 1991. Detection of HSV-1 DNA in patients with Behc ̧et's syndrome and in patients with recurrent oral ulcers by the polymerase chain reaction. J Med Microbiol. 34:39–43.

140. Mendoza-Pinto C, Garcı a-Carrasco M, Jime nez-Herna ndez M, Hernandez C, Riebeling-Navarro C, Zavala A, Recabarren M, Espinosa G, Quezada J, Cervera R. 2010. Etiopathogenesis of Behcet's disease. Autoimmun Rev. 9:241–245.

141. Van Der Houwen TB, Dik WA, Goeijenbier M, Hayat M, Nagtzaam NMA, Van Hagen M, et al. Leukocyte toll-like receptor expression in pathergy positive and negative Behcet's disease patients. Rheumatology 2020.

142. Kim EH, Park MJ, Park S, Lee ES. Increased expression of the NLRP3 inflammasome components in patients with Beh¸cet'sdisease.JInflamm 2015;12 (1):41.

143. Ureten K, Ertenli I, Ozt€ urk MA, Kiraz S, Onat AM, Tuncer M, et al. Neutrophil CD64 expression in Beh¸cet's disease. J Rheumatol 2005;32(5):849–52.

144. Hedayatfar, A., 2013. Behçet's Disease: Autoimmune or Autoinflammatory? J Ophthalmic Vis Res 8, 291–293.

145. Hamzaoui K, Bouali E, Ghorbel I, Khanfir M, Houman H, Hamzaoui A. Expression of Th-17 and RORgt mRNA in Behçet's disease. Med Sci Monit 2011;17:CR227-234.

146. Evereklioglu C. 2005. Current concepts in the etiology and treatment of Behc¸et disease. Surv Ophthalmol. 50:297–350.

147. Harzallah, O., Kerkeni, A., Baati, T., Mahjoub, S., 2008. Oxidative stress: Correlation with Behçet's disease duration, activity and severity. European Journal of Internal Medicine 19, 541–547

148. Köse K, Dogan P, Ascioglu M, Erkiliç K, Ascioglu Ö. Oxidative stress and antioxidant defenses in plasma of patients with Behçet's disease. Tohoku J Exp Med 1995;176:239–48.

149. Taysi S, Demircan B, Akdeniz N, Atasoy M, Sari RA. Oxidant/antioxidant status in men with Behçet's disease. Clin Rheumatol 2007;26:418–22

150. Becatti, M., Emmi, G., Silvestri, E., Bruschi, G., Ciucciarelli, L., Squatrito, D., Vaglio, A., Taddei, N., Abbate, R., Emmi, L., Goldoni, M., Fiorillo, C., Prisco, D., 2016. Neutrophil Activation Promotes Fibrinogen Oxidation and Thrombus Formation in Behçet Disease. Circulation 133, 302–311.

151. Emmi, G., Becatti, M., Bettiol, A., Hatemi, G., Prisco, D., Fiorillo, C., 2019. Behçet's Syndrome as a Model of Thrombo-Inflammation: The Role of Neutrophils. Frontiers in Immunology 10.

152. Chaturvedi, S., McCrae, K.R., 2017. Diagnosis and management of the antiphospholipid syndrome. Blood Rev 31, 406–417.

153. Petri, M., 2020. Antiphospholipid syndrome. Transl Res 225, 70–81.

154. Miyakis S, Lockshin MD, Atsumi T, Branch DW, Brey RL, Cervera R, et al. International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). J Thromb Haemost. 2006; 4:295–306.

155. Belizna, C., Stojanovich, L., Cohen-Tervaert, J.W., Fassot, C., Henrion, D., Loufrani, L., Nagy, G., Muchardt, C., Hasan, M., Ungeheuer, M.N., Arnaud, L., Alijotas-Reig, J., Esteve-Valverde, E., Nicoletti, F., Saulnier, P., Godon, A., Reynier, P., Chrétien, J.M., Damian, L., Omarjee, L., Mahé, G., Pistorius, M.A., Meroni, P.L., Devreese, K., 2018. Primary antiphospholipid syndrome and antiphospholipid syndrome associated to systemic lupus: Are they different entities? Autoimmun Rev 17, 739–745.

156. Asherson RA, Cervera R. 'Primary', 'secondary' and other variants of the antiphospholipid syndrome. Lupus 1994;3:293-8.

157. R. Cervera, R. Serrano, G.J. Pons-Estel, L. Ceberio-Hualde, Y. Shoenfeld, E. De Ramón, V. Buonaiuto, S. Jacobsen, M.M. Zeher, T. Tarr, A. Tincani, M. Taglietti, G. Theodossiades, E. Nomikou, M. Galeazzi, F. Bellisai, P.L. Meroni, R.H.W.M. Derksen, P.G.D. De Groot, M. Baleva, S. Mosca, M. Bombardieri, F. Houssiau, J.C. Gris, I. Quéré, E. Hachulla, C. Vasconcelos, A. Fernández-Nebro, M. Haro, Z. Amoura, M. Miyara, M. Tektonidou, G. Espinosa, M.L. Bertolaccini, M.A. Khamashta, Morbidity and mortality in the antiphospholipid syndrome during a 10-year period: A multicentre prospective study of 1000 patients, Ann Rheum Dis. 74 (2015) 1011–1018. https://doi.org/10.1136/annrheumdis-2013-204838.

158. Negrini, S., Pappalardo, F., Murdaca, G., Indiveri, F., Puppo, F., 2017. The antiphospholipid syndrome: from pathophysiology to treatment. Clin Exp Med 17, 257–267.

159. Ortiz-Fernández, L., Sawalha, A.H., 2019. Genetics of Antiphospholipid Syndrome. Curr Rheumatol Rep 21, 65.

160. Iuliano, A., Galeazzi, M., Sebastiani, G.D., 2019. Antiphospholipid syndrome's genetic and epigenetic aspects. Autoimmunity Reviews 18, 102352. https://doi.org/10.1016/j.autrev.2019.102352

161. Kapitany A, Tarr T, Gyetvai A, Szodoray P, Tumpek J, Poor G et al. Human leukocyte antigen-DRB1 and -DQB1 genotyping in lupus patients with and without antiphospholipid syndrome. Ann N Y Acad Sci 2009;1173:545-51.

162. Freitas MV, da Silva LM, Deghaide NH, Donaldi EA, Louzada-Junior P. Is HLA class II susceptibility to primary antiphospholipid syndrome different from susceptibility to secondary antiphospholipid syndrome? Lupus 2004; 13:125–31.

163. Yasuda S,Atsumi T,Matsuura E,Kaihara K,Yamamoto D, Ichikawa K, et al. Significance of valine/leucine247 polymorphism of beta2-glycoprotein I in antiphospholipid syndrome: increased reactivity of anti-beta2-glycoprotein I autoantibodies to the valine247 beta2-glycoprotein I variant. Arthritis Rheum. 2005;52: 212–8

164. Horita T, Atsumi T, Yoshida N, Nakagawa H, Kataoka H, Yasuda S, et al. STAT4 single nucleotide polymorphism, rs7574865 G/T, as a risk for antiphospholipid syndrome. Ann Rheum Dis. 2009;68: 1366–7.

165. Wasserman A. Uber Entwicklung und den Gegenwartingen Stand der Serodiagnostic Gegenuber Syphilis. Berl Klin Wochenschr 1907; 44: 1599–1634.

166. Santiago M, Martinelli R, Ko A et al. Anti-beta2 glycoprotein I and anticardiolipin antibodies in leptospirosis, syphilis and Kala-azar. Clin Exp Rheumatol 2001; 19: 425–430.

167. Consigny PH, Cauquelin B, Agnamey P et al. High prevalence of cofactor independent anticardiolipin antibodies in malaria exposed individuals. Clin Exp Immunol 2002; 127: 158–164.

168. Cicconi V, Carloni E, Franceschi F et al. Disappearance of antiphospolipid antibodies syndrome after Helicobacter pylori eradication. Am J Med 2001; 111: 163–164.

169. Elefsiniotis IS, Diamantis ID, Dourakis SP, Kafiri G, Pantazis K, Mavrogiannis C. Anticardiolipin antibodies in chronic hepatitis B and chronic hepatitis D infection, and hepatitis B-related hepatocellular carcinoma. Relationship with portal vein thrombosis. Eur J Gastroenterol Hepatol 2003; 15: 721–726.

170. van Hal S, Senanayake S, Hardiman R. Splenic infarction due to transient antiphospholipid antibodies induced by acute Epstein-Barr virus infection. J Clin Virol 2005; 32: 245–247.

171. Jaeger U, Kapiotis S, Pabinger I, Puchhammer E, Kyrle PA, Lechner K. Transient lupus anticoagulant associated with hypoprothrombinemia and factor XII deficiency following adenovirus infection, Ann Hematol 1993; 67: 95–99.

172. Levy, Y., Almog, O., Gorshtein, A., Shoenfeld, Y., 2006. The environment and antiphospholipid syndrome. Lupus 15, 784–790. https://doi.org/10.1177/0961203306071004

173. Ferraro-Peyret C, Coury F, Tebib JG, Bienvenu J, Fabien N. Infliximab therapy in rheumatoid arthritis and ankylosing spondylitis-induced specific antinuclear and antiphospholipid autoantibodies without autoimmune clinical manifestations: a two-year prospective study. Arthritis Res Ther 2004; 6: R535–543.

174. Meroni PL, Borghi MO, Raschi E, Tedesco F. Pathogenesis of antiphospholipid syndrome: understanding the antibodies. Nat Rev Rheumatol. 2011 Jun;7(6):330-9. doi: 10.1038/nrrheum.2011.52. Epub 2011 May 10. PMID: 21556027.

175. Knight, J.S., Branch, D.W., Ortel, T.L., 2023. Antiphospholipid syndrome: advances in diagnosis, pathogenesis, and management. BMJ 380, e069717.

176. Nocella, C., Bartimoccia, S., Cammisotto, V., D'Amico, A., Pastori, D., Frati, G., Sciarretta, S., Rosa, P., Felici, C., Riggio, O., Calogero, A., Carnevale, R., SMiLe Group, null, 2021. Oxidative Stress in the Pathogenesis of Antiphospholipid Syndrome: Implications for the Atherothrombotic Process. Antioxidants (Basel) 10, 1790.

177. Davatchi, F., Chams-Davatchi, C., Shams, H., Shahram, F., Nadji, A., Akhlaghi, M., Faezi, T., Ghodsi, Z., Sadeghi Abdollahi, B., Ashofteh, F., Mohtasham, N., Kavosi, H., Masoumi, M., 2017. Behcet's disease: epidemiology, clinical manifestations, and diagnosis. Expert Rev Clin Immunol 13, 57–65.

178. van der Woude, D., van der Helm-van Mil, A.H.M., 2018. Update on the epidemiology, risk factors, and disease outcomes of rheumatoid arthritis. Best Pract Res Clin Rheumatol 32, 174–187.

179. Lim, S.S., Drenkard, C., 2015. Epidemiology of lupus: an update. Curr Opin Rheumatol 27, 427–432.

180. Calderon, L.M., Pope, J.E., 2021. Scleroderma epidemiology update. Curr Opin Rheumatol 33, 122–127

181. Dabit, J.Y., Valenzuela-Almada, M.O., Vallejo-Ramos, S., Duarte-García, A., 2022. Epidemiology of Antiphospholipid Syndrome in the General Population. Curr Rheumatol Rep 23, 85.

182. Martelli-Palomino, G., Paoliello-Paschoalato, A.B., Crispim, J.C.O., Rassi, D.M., Oliveira, R.D., Louzada, P., Lucisano-Valim, Y.M., Donadi, E.A., 2017. DNA damage increase in peripheral neutrophils from patients with rheumatoid arthritis is associated with the disease activity and the presence of shared epitope. Clin Exp Rheumatol 35, 247–254.

183. Altindag, O., Karakoc, M., Kocyigit, A., Celik, H., Soran, N., 2007. Increased DNA damage and oxidative stress in patients with rheumatoid arthritis. Clin Biochem 40, 167–171.

184. McConnell, J.R., Crockard, A.D., Cairns, A.P., Bell, A.L., 2002. Neutrophils from systemic lupus erythematosus patients demonstrate increased nuclear DNA damage. Clin Exp Rheumatol 20, 653–660.

185. Kaplan, M.J., 2011. Neutrophils in the pathogenesis and manifestations of SLE. Nat Rev Rheumatol 7, 691–699.

186. Sambo, P., Baroni, S.S., Luchetti, M., Paroncini, P., Dusi, S., Orlandini, G., Gabrielli, A., 2001. Oxidative stress in scleroderma: maintenance of scleroderma fibroblast phenotype by the constitutive up-regulation of reactive oxygen species generation through the NADPH oxidase complex pathway. Arthritis Rheum 44, 2653–2664.

187. Tsou, P.-S., Talia, N.N., Pinney, A.J., Kendzicky, A., Piera-Velazquez, S., Jimenez, S.A., Seibold, J.R., Phillips, K., Koch, A.E., 2012. Effect of oxidative stress on protein tyrosine phosphatase 1B in scleroderma dermal fibroblasts. Arthritis Rheum 64, 1978–1989.

188. Servettaz, A., Goulvestre, C., Kavian, N., Nicco, C., Guilpain, P., Chéreau, C., Vuiblet, V., Guillevin, L., Mouthon, L., Weill, B., Batteux, F., 2009. Selective oxidation of DNA topoisomerase 1 induces systemic sclerosis in the mouse. J Immunol 182, 5855–5864.

189. Buldanlioglu, S., Turkmen, S., Ayabakan, H.B., Yenice, N., Vardar, M., Dogan, S., Mercan, E., 2005. Nitric oxide, lipid peroxidation and antioxidant defence system in patients with active or inactive Behçet's disease. Br J Dermatol 153, 526–530.

190. Karaman, A., Kadi, M., Kara, F., 2009. Sister chromatid exchange and micronucleus studies in patients with Behçet's disease. J Cutan Pathol 36, 831–837.

191. Delgado Alves, J., Mason, L.J., Ames, P.R.J., Chen, P.P., Rauch, J., Levine, J.S., Subang, R., Isenberg, D.A., 2005. Antiphospholipid antibodies are associated with enhanced oxidative stress, decreased plasma nitric oxide and paraoxonase activity in an experimental mouse model. Rheumatology (Oxford) 44, 1238–1244.

192. Alves, J.D., Ames, P.R.J., 2003. Atherosclerosis, oxidative stress and auto-antibodies in systemic lupus erythematosus and primary antiphospholipid syndrome. Immunobiology 207, 23–28.

193. López-Otín, C., Blasco, M.A., Partridge, L., Serrano, M., Kroemer, G., 2013. The Hallmarks of Aging. Cell 153, 1194–1217.

194. Luo, J., Mills, K., le Cessie, S., Noordam, R., van Heemst, D., 2020. Ageing, age-related diseases and oxidative stress: What to do next? Ageing Res Rev 57, 100982.

195. Milic, M., Frustaci, A., Del Bufalo, A., Sánchez-Alarcón, J., Valencia-Quintana, R., Russo, P., Bonassi, S., 2015. DNA damage in non-communicable diseases: A clinical and epidemiological perspective. Mutat Res 776, 118–127.

196. Møller, P., Stopper, H., Collins, A.R., 2020. Measurement of DNA damage with the comet assay in high-prevalence diseases: current status and future directions. Mutagenesis 35, 5–18.

197. Bonassi, S., Ceppi, M., Møller, P., Azqueta, A., Milić, M., Neri, M., Brunborg, G., Godschalk, R., Koppen, G., Langie, S.A.S., Teixeira, J.P., Bruzzone, M., Da Silva, J., Benedetti, D., Cavallo, D., Ursini, C.L., Giovannelli, L., Moretti, S., Riso, P., Del Bo’, C., Russo, P., Dobrzyńska, M., Goroshinskaya, I.A., Surikova, E.I., Staruchova, M., Barančokova, M., Volkovova, K., Kažimirova, A., Smolkova, B., Laffon, B., Valdiglesias, V., Pastor, S., Marcos, R., Hernández, A., Gajski, G., Spremo-Potparević, B., Živković, L., Boutet-Robinet, E., Perdry, H., Lebailly, P., Perez, C.L., Basaran, N., Nemeth, Z., Safar, A., Dusinska, M., Collins, A., hCOMET project, 2021. DNA damage in circulating leukocytes measured with the comet assay may predict the risk of death. Sci Rep 11, 16793.

198. Milić, M., Ceppi, M., Bruzzone, M., Azqueta, A., Brunborg, G., Godschalk, R., Koppen, G., Langie, S., Møller, P., Teixeira, J.P., Alija, A., Anderson, D., Andrade, V., Andreoli, C., Asllani, F., Bangkoglu, E.E., Barančoková, M., Basaran, N., Boutet-Robinet, E., Buschini, A., Cavallo, D., Costa Pereira, C., Costa, C., Costa, S., Da Silva, J., Del Bo´, C., Dimitrijević Srećković, V., Djelić, N., Dobrzyńska, M., Duračková, Z., Dvořáková, M., Gajski, G., Galati, S., García Lima, O., Giovannelli, L., Goroshinskaya, I.A., Grindel, A., Gutzkow, K.B., Hernández, A., Hernández, C., Holven, K.B., Ibero-Baraibar, I., Ottestad, I., Kadioglu, E.,

Kažimirová, A., Kuznetsova, E., Ladeira, C., Laffon, B., Lamonaca, P., Lebailly, P., Louro, H., Mandina Cardoso, T., Marcon, F., Marcos, R., Moretti, M., Moretti, S., Najafzadeh, M., Nemeth, Z., Neri, M., Novotna, B., Orlow, I., Paduchova, Z., Pastor, S., Perdry, H., Spremo-Potparević, B., Ramadhani, D., Riso, P., Rohr, P., Rojas, E., Rossner, P., Safar, A., Sardas, S., Silva, M.J., Sirota, N., Smolkova, B., Staruchova, M., Stetina, R., Stopper, H., Surikova, E.I., Ulven, S.M., Ursini, C.L., Valdiglesias, V., Valverde, M., Vodicka, P., Volkovova, K., Wagner, K.-H., Živković, L., Dušinská, M., Collins, A.R., Bonassi, S., 2021. The hCOMET project: International database comparison of results with the comet assay in human biomonitoring. Baseline frequency of DNA damage and effect of main confounders. Mutat Res Rev Mutat Res 787, 108371.

199. Garm, C., Moreno-Villanueva, M., Bürkle, A., Petersen, I., Bohr, V.A., Christensen, K., Stevnsner, T., 2013. Age and gender effects on DNA strand break repair in peripheral blood mononuclear cells. Aging Cell 12, 58–66.

200. Mirończuk-Chodakowska, I., Witkowska, A.M., Zujko, M.E., 2018. Endogenous non-enzymatic antioxidants in the human body. Advances in Medical Sciences 63, 68–78.

201. Wu, J.Q., Kosten, T.R., Zhang, X.Y., 2013. Free radicals, antioxidant defense systems, and schizophrenia. Progress in Neuro-Psychopharmacology and Biological Psychiatry 46, 200–206.

202. Babula, P., Masarik, M., Adam, V., Eckschlager, T., Stiborova, M., Trnkova, L., Skutkova, H., Provaznik, I., Hubalek, J., Kizek, R., 2012. Mammalian metallothioneins: properties and functions. Metallomics 4, 739–750.

203. Poetsch, A.R., 2020. The genomics of oxidative DNA damage, repair, and resulting mutagenesis. Computational and Structural Biotechnology Journal 18, 207–219.

204. Mittal, M., Siddiqui, M.R., Tran, K., Reddy, S.P., Malik, A.B., 2014. Reactive Oxygen Species in Inflammation and Tissue Injury. Antioxidants & Redox Signaling 20, 1126–1167.