NATIONAL AND **K**APODISTRIAN **U**NIVERSITY OF **A**THENS

SCHOOL OF **M**EDICINE

DEPARTMENT OF SURGERY |
LABORATORY OF EXPERIMENTAL SURGERY &
SURGICAL RESEARCH "N.S. CHRISTEAS"
DIRECTOR: PROF. KONSTANTINOS KONTZOGLOU

THE IMPACT OF ANTI-INFLAMMATORY TREATMENT ON THE BIOCHEMISTRY AND STEM CELL GENETICS OF ATHEROMATOSIS

PhD DISSERTATION

Konstantinos S. Mylonas, M.D.

Supervisor

Nikolaos Nikiteas | Professor of General Surgery, National and Kapodistrian University of Athens

ATHENS 2022

This research was funded by the Hellenic Surgical Society and the Hellenic Atherosclerosis Society.

ΕΘΝΙΚΟ ΚΑΙ **Κ**ΑΠΟΔΙΣΤΡΙΑΚΟ **Π**ΑΝΕΠΙΣΤΗΜΙΟ **Α**ΘΗΝΩΝ

ΙΑΤΡΙΚΗ **Σ**ΧΟΛΗ

ΧΕΙΡΟΥΡΓΙΚΟΣ ΤΟΜΕΑΣ |
ΕΡΓΑΣΤΗΡΙΟ ΠΕΙΡΑΜΑΤΙΚΗΣ ΧΕΙΡΟΥΡΓΙΚΗΣ &
ΧΕΙΡΟΥΡΓΙΚΗΣ ΕΡΕΥΝΑΣ «Ν.Σ. ΧΡΗΣΤΕΑΣ»
ΔΙΕΥΘΥΝΤΗΣ: ΚΑΘΗΓΗΤΗΣ ΚΩΝΣΤΑΝΤΙΝΟΣ ΚΟΝΤΖΟΓΛΟΥ

ΦΑΡΜΑΚΟΛΟΓΙΚΕΣ ΕΠΙΔΡΑΣΕΙΣ ΣΤΗ ΜΟΡΙΑΚΗ ΒΙΟΧΗΜΕΙΑ ΤΗΣ ΑΘΗΡΩΜΑΤΩΣΗΣ: ΜΕΛΕΤΗ ΕΠΙ ΠΕΙΡΑΜΑΤΙΚΟΥ ΜΟΝΤΕΛΟΥ

ΔΙΔΑΚΤΟΡΙΚΗ ΔΙΑΤΡΙΒΗ

ΚΩΝΣΤΑΝΤΙΝΟΣ Σ. ΜΥΛΩΝΑΣ

ΙΑΤΡΟΣ

Επιβλέπων

Νικόλαος Νικητέας | Καθηγητής Γενικής Χειρουργικής, Εθνικό και Καποδιστριακό Πανεπιστήμιο Αθηνών

ΑΘΗΝΑ 2022

Η παρούσα έρευνα χρηματοδοτήθηκε από την Ελληνική Χειρουργική Εταιρεία και την Ελληνική Εταιρεία Αθηροσκλήρωσης.

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The views and conclusions contained in this document express the author and should not be construed as representing the official positions of the National and Kapodistrian University of Athens.

Στοιχεία Διδακτορικής Διατριβής

Αίτηση εκπόνησης διδακτορικής διατριβής | 25.05.2018

Αριθμός πρωτοκόλλου: 1718029198

Ορισμός τριμελούς συμβουλευτικής επιτροπής | 04.10.2018

Αριθμός πρωτοκόλλου: 1819004350

Ορισμός θέματος διδακτορικής διατριβής | 14.01.2019

Αριθμός πρωτοκόλλου: 066

Κατάθεση διδακτορικής διατριβής | 23.11.2022

Αριθμός πρωτοκόλλου: 126224

Ορισμός επταμελούς εξεταστικής επιτροπής | 01.12.2022

Αριθμός πρωτοκόλλου: 129588

ADVISORY COMMITTEE

Nikolaos Nikiteas| Professor of General Surgery (Supervisor)

National and Kapodistrian University of Athens

Theodoros Liakakos| Professor of General Surgery

National and Kapodistrian University of Athens

Dimitrios Iliopoulos| Associate Professor of Cardiac Surgery

National and Kapodistrian University of Athens

Professor Theodoros Liakakos was replaced by Assistant Professor Dimitrios Schizas following his retirement (16.07.2020/1920030842)

EXAMINATION COMMITTEE

Nikolaos Nikiteas| Professor of General Surgery (Supervisor)

National and Kapodistrian University of Athens

Dimitrios Iliopoulos| Associate Professor of Cardiac Surgery

National and Kapodistrian University of Athens

Dimitrios Schizas| Assistant Professor of General Surgery

National and Kapodistrian University of Athens

Konstantinos Kontzoglou| Professor of General Surgery

National and Kapodistrian University of Athens

Dimitrios Angouras| Professor of Cardiac Surgery

National and Kapodistrian University of Athens

Christos Bakoyiannis| Professor of Vascular Surgery

National and Kapodistrian University of Athens

Gerasimos Siasos| Professor of Cardiology-Molecular Cardiology

National and Kapodistrian University of Athens

ΤΡΙΜΕΛΗΣ ΣΥΜΒΟΥΛΕΥΤΙΚΗ ΕΠΙΤΡΟΠΗ

Νικόλαος Νικητέας| Καθηγητής Γενικής Χειρουργικής (Επιβλέπων)
Εθνικό και Καποδιστριακό Πανεπιστήμιο Αθηνών

Θεόδωρος Λιακάκος| Καθηγητής Γενικής Χειρουργικής
Εθνικό και Καποδιστριακό Πανεπιστήμιο Αθηνών

Δημήτριος Ηλιόπουλος| Αναπληρωτής Καθηγητής Καρδιοχειρουργικής
Εθνικό και Καποδιστριακό Πανεπιστήμιο Αθηνών

Ο Καθηγητής Θεόδωρος Λιακάκος αντικαταστάθηκε λόγω αφυπηρέτησης από τον Επίκουρο Καθηγητή Δημήτριο Σχίζα (16.07.2020/1920030842)

ΕΠΤΑΜΕΛΗΣ ΕΞΕΤΑΣΤΙΚΗ ΕΠΙΤΡΟΠΗ

Νικόλαος Νικητέας| Καθηγητής Γενικής Χειρουργικής (Επιβλέπων)
Εθνικό και Καποδιστριακό Πανεπιστήμιο Αθηνών

Δημήτριος Ηλιόπουλος| Αναπληρωτής Καθηγητής Καρδιοχειρουργικής
Εθνικό και Καποδιστριακό Πανεπιστήμιο Αθηνών

Δημήτριος Σχίζας| Επίκουρος Καθηγητής Γενικής Χειρουργικής
Εθνικό και Καποδιστριακό Πανεπιστήμιο Αθηνών

Κωνσταντίνος Κόντζογλου| Καθηγητής Γενικής Χειρουργικής
Εθνικό και Καποδιστριακό Πανεπιστήμιο Αθηνών

Δημήτριος Αγγουράς| Καθηγητής Καρδιοχειρουργικής
Εθνικό και Καποδιστριακό Πανεπιστήμιο Αθηνών

Χρήστος Μπακογιάννης| Καθηγητής Αγγειοχειρουργικής
Εθνικό και Καποδιστριακό Πανεπιστήμιο Αθηνών

Γεράσιμος Σιάσος| Καθηγητής Καρδιολογίας-Μοριακής Καρδιολογίας
Εθνικό και Καποδιστριακό Πανεπιστήμιο Αθηνών

Αφιέρωση

Στην οικογένειά μου με αγάπη,

Στους Δασκάλους μου με ευγνωμοσύνη,

Ο ΟΡΚΟΣ ΤΟΥ ΙΠΠΟΚΡΑΤΗ

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 ποήσασθαι, καὶ γένος τὸ ἐξ αὐτέου ἀδελφοῖς ἴσον ἐπικρινέειν ἄρρεσι, καὶ διδάξειν τὴν τέχνην ταύτην, ἢν χρηίζωσι μανθάνειν, ἄνευ μισθοῦ καὶ ξυγγραφῆς, παραγγελίης τε καὶ ἀκροήσιος καὶ τῆς λοιπῆς ἁπάσης μαθήσιος μετάδοσιν ποήσασθαι 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, ἐ λευθέρων 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 παρ ἁπᾶσιν ἀνθρώποις ἐς τὸν αἰεὶ χρόνον. παραβαίνοντι δὲ καὶ ἐπιορκοῦντι, τἀναντία τουτέων

Contents

Acknowledgments

The present doctoral dissertation was conducted at the Laboratory of Experimental Surgery and Surgical Research "*N.S. Christeas*", School of Medicine, National and Kapodistrian University of Athens.

First and foremost, I would like to express my sincere gratitude and appreciation to my advisory committee, *Professor Nikolaos Nikiteas*, *Associate Professor Dimitrios Iliopoulos,* and *Assistant Professor Dimitrios Schizas,* for their guidance and unwavering support during the course of this doctoral research. *Professor Theodore Liakakos* was a member of my original advisory committee prior to retiring. His decades of experience and wisdom were key during the early phases of project design.

Professor Konstantinos Kontzoglou graciously welcomed me to the Laboratory of Experimental Surgery and Surgical Research "*N.S. Christeas*" and streamlined the completion of the present experiment. Furthermore, I would like to thank the personnel of the lab, especially *Mr. Panagiotis Tsakiropoulos* and *Mr. Nikolaos Tsakiropoulos*, for their administrative support and assistance with animal maintenance.

Credit should also be given to *Dr. Panagiotis Sarantis* (Department of Biological Chemistry, School of Medicine, National and Kapodistrian University of Athens) and *Dr. Alkistis Kapelouzou* (Department of Clinical, Experimental Surgery & Translational Research, Biomedical Research Foundation, Academy of Athens). Their role was decisive in fine-tuning the experiment and completing the biochemical analyses.

It should also be emphasized that the present doctoral research was funded by the *Hellenic Surgical Society* and the *Hellenic Atherosclerosis Society*. Obtaining many of the key resources that were used in the present experiment would not have been possible without these generous grants.

Last but not least, to *Dr. Peter Masiakos* (Associate Professor of Surgery, Harvard

Medical School) I probably owe an entire letter of appreciation. Dr. Masiakos mentored my early research years and instilled the principles of meaningful research. He spent countless hours molding me into not only a stronger scientist but also into a better person. He taught me humility and made me realize that for all our wisdom, there is still so much more we don't know.

Konstantinos S. Mylonas, MD, PhD

Doctoral scientific output

Publications

Original research

1. **Mylonas K.S.,** Sarantis P., Kapelouzou A., Karamouzis M., Iliopoulos D.C., Nikiteas N., Schizas D. ***The aortic expression pattern of mechanosensitive stem cell genes is spatially deranged by western-type diet but can be regulated with colchicine-based anti-inflammatory treatment.*** European Heart Journal. Volume 43 (s2), October 2022, ehac544.3053

Impact factor: 35.9

2. **Mylonas K.S.,** Sarantis P., Kapelouzou A., Karamouzis M., Kapetanakis E.I., Kontzoglou K., Iliopoulos D.C., Nikiteas N., Schizas D. ***Mechanosensitive Stem Cell Genes and Klotho in Atherosclerotic Aortas: Regulating Expression Patterns using Colchicine Regimens.*** Journal of Clinical Medicine. October 2022. 11(21), 6465

Impact factor: 5.1

3. **Mylonas K.S.,** Kapelouzou A., Spartalis M.D, Mastrogeorgiou M., Spartalis E.D., Bakoyiannis C., Liakakos T., Schizas D., Nikiteas N., Iliopoulos D.C. ***KLF4 Upregulation in Atherosclerotic Thoracic Aortas: Exploring the Protective Effect of Colchicine-based Regimens in a Hyperlipidemic Rabbit Model***. Annals of Vascular Surgery. 26/6/21: S0890-5096(21)00436-2.

Impact factor: 1.6

4. **Mylonas K.S.*,** Bakoyiannis C.*, Papoutsis K., Kakavia K., Schizas D., Iliopoulos D.C., Nikiteas N., Liakakos T., Kapelouzou A. ***Increased Serum KLF4 in Severe Atheromatosis and Extensive Aneurysmal Disease***. Annals of Vascular Surgery. 2020 Oct;68:338-343.

Impact factor: 1.6

5. Spartalis M.D, Siasos G., Mastrogeorgiou M., Spartalis E., Kaminiotis V., **Mylonas K.S.,** Kapelouzou A., Kontogiannis C., Doulamis I.P, Toutouzas K., Nikiteas N., Iliopoulos D.C.

The effect of per os colchicine administration in combination with fenofibrate and N-acetylcysteine on triglyceride levels and the development of atherosclerotic lesions in cholesterol-fed rabbits. European Review for Medical and Pharmacological Sciences. 2021 Dec;25(24):7765-7776.

Impact factor: 3.5

**co-first authors*

Reviews

6. **Mylonas K.S.,** Karangelis D., Androutsopoulou V., Chalikias G., Tziakas D., Mikroulis D., Iliopoulos D.C., Nikiteas N., Schizas D. ***Stem Cell Genes in Atheromatosis: The role of Klotho, HIF1α, OCT4, and BMP4.*** IUBMB Life. 2022 Oct;74(10):1003-1011.

Impact factor: 4.7

7. **Mylonas K.S.,** Iliopoulos D.C., Nikiteas N., Schizas D. ***Looking for The Achilles Heel of Atheromatosis: Could It Be Immunotherapy?*** Immunotherapy. 2021 May;13(7):557-560.

Impact factor: 4.1

8. **Mylonas K.S.,** Kapelouzou A., Spartalis M., Schizas D., Spartalis E., Bakoyiannis C. Iliopoulos D.C., Liakakos T., Nikiteas N. ***Inflammation and Immunotherapy in Atherosclerosis***. Journal of Atherosclerosis Prevention and Treatment. 2020 May-Aug;11(2):67–75

Impact factor: - (Requested in the context of the scholarship by the Hellenic Atherosclerosis Society)

Podium presentations in international meetings

European Society of Cardiology Congress 2022 (August 2022, Barcelona, Spain)

1. **Mylonas K.S.,** Sarantis P., Kapelouzou A., Karamouzis M., Iliopoulos D.C., Nikiteas N., Schizas D. The aortic expression pattern of mechanosensitive stem cell genes is spatially deranged by western-type diet but can be regulated with colchicine-based anti-inflammatory treatment

Podium presentations in national meetings

14th Panhellenic Congress of the Hellenic Society of Thoracic and Cardiovascular Surgeons (November 2022, Thessaloniki, Greece)

2. **Mylonas K.S.,** Sarantis P., Kapelouzou A., Karamouzis M., Iliopoulos D.C., Nikiteas N., Schizas D. Developmental Genes and Klotho in Aortic Atherosclerosis: Addressing Multilevel Dysregulation using Colchicine Regimens

10th Annual Congress of the Hellenic Atherosclerosis Society (Nov-Dec 2022, Athens, Greece)

2. **Mylonas K.S.,** Sarantis P., Kapelouzou A., Karamouzis M., Iliopoulos D.C., Nikiteas N., Schizas D. Aortic expression of mechanosensitive developmental genes is regionally dysregulated by western-type diet but can be regulated with colchicine-based regimens

9th Annual Congress of the Hellenic Atherosclerosis Society (December 2020, Athens, Greece)

3. **Mylonas K.S.,** Kapelouzou A., Spartalis M.D., Mastrogeorgiou M., Spartalis E.D., Bakoyiannis C., Liakakos T., Schizas D., Nikiteas N., Iliopoulos D.C. Combining colchicine with clofibrate or N-acetylcysteine curtails the development of thoracic aortic atheromatosis in an experimental rabbit model

40th Panhellenic Cardiology Congress (October 2019, Athens, Greece)

4. Spartalis M.D, Mastrogeorgiou M., **Mylonas K.S.,** Tzatzaki E., Spartalis E.D., Nikiteas N., Iliopoulos D.C. Effect of colchicine with clofibrate or N-acetylcysteine on triglyceride levels and atheromatosis plaques in an experimental rabbit model

Doctoral scholarships

- February 2020 – Hellenic Surgical Society (Johnson & Johnson)
- December 2019 – Hellenic Atherosclerosis Society

Congress awards

- December 2022 – Third Award in the Oral Presentations section
  10thAnnual Congress of the Hellenic Atheromatosis Society
  Awarded by the Hellenic Atherosclerosis Society

Curriculum Vitae

Konstantinos S. Mylonas, MD

CONTACT DETAILS

Nationality: Greek
Marital status: Single
Cellphone number: (+30) 6987024653
Personal email: ksmylonas@gmail.com

CURRENT POSITION

August 2021- **Onassis Cardiac Surgery Center**
Cardiac Surgery Resident
Cardiac Surgeon-in-Chief: George Stavridis, MD, FETCS
Program director: Dimitrios V. Avgerinos, MD, PhD, FACC, FACS

PRIOR WORK EXPERIENCE

May 2019-Aug 2021 **Laikon General Hospital, University of Athens**
Surgeon-In-Chief: Professor Evangelos Felekouras, MD, PhD
General Surgery Resident
Track*: Cardiothoracic Surgery*
Served as chief resident on the *Transplant Surgery service*

Nov 2018-April 2019 **Mitera Children's Hospital**
Senior House Officer in Pediatric Cardiovascular Surgery
Cardiac Surgeon-In-Chief: Professor Afksendiyos Kalangos, MD, PhD

Jan 2017-Oct 2018 **Massachusetts General Hospital, Harvard Medical School**
Supervisors: Peter T. Masiakos, MD, MS, FACS, FAAP & Cassandra M. Kelleher, MD, FACS

June 2018-Oct 2018 Graduate Trainee & HMS Teaching Clinical Fellow in Pediatrics
Rotations: *Pediatric Surgery, Pediatric Trauma & Emergency Medicine*

Jan 2017-May 2018 Postdoctoral Research Fellowship in Pediatric Surgery
Fully funded position

EDUCATION

Oct 2018-ongoing **National and Kapodistrian University of Athens, School of Medicine**
PhD Candidate in Cardiovascular Stem Cell Genetics
Thesis: *The impact of anti-inflammatory treatment on the biochemistry and stem cell genetics of atheromatosis*
Experimental animal model and genes of interest:

- *White New Zealand rabbit*
- *KLF4, Klotho, HOXA5, HIF1α, NOTCH1, BMP4, OCT4, SOX2, NANOG*

Supervisors: Nikolaos Nikiteas, MD, PhD
Grant support*: i) Hellenic Surgical Society (Johnson & Johnson), ii) Hellenic Atherosclerosis Society*

Sep 2017-July 2018 **Harvard Medical School**
PDCP in Applied Biostatistics
Program Director: Brian Healy, PhD

Sep 2010-Oct 2016 **Aristotle University of Thessaloniki, School of Medicine**
Six-year M.D. program
Class Valedictorian (1st out of 366 graduates)
Summa Cum Laude
GPA: 9.29/10

Away rotations performed as a visiting sub-intern during the academic year 2015-2016

July 2016 **NIH Clinical Center**
Pediatrics sub-internship
Preceptor: David Lang, MD, MPH
Final grade: Honors

January 2016 **Boston Children's Hospital**
Pediatric Surgery sub-internship
Preceptor: Craig Lillehei, MD
Final grade: Honors

Nov-Dec 2015 **Memorial Sloan Kettering Cancer Center**

Breast Surgical Oncology sub-internship
Preceptor: Virgillio Sacchini, MD
Final grade: Pass (on a Pass/Fail scale)

July-August 2015 **Mayo Clinic School of Graduate Medical Education**
General Surgery sub-internship
Preceptor: Rory Smoot, MD
Final grade: Honors

UNDERGRADUATE EDUCATION

Oct 2007-June 2010 **4th High School of Trikala, "Alexander Papadiamantis"**
Class Valedictorian *(GPA: 19.8/20)*

HONORS & AWARDS

December 2022 Third Award in the Oral Presentations Section
10th Annual Congress of the Hellenic Atherosclerosis Society

November 2022 Young Investigator Award in Aortic Valve Repair
Awarded by the Hellenic Society of Thoracic and CV Surgeons

February 2020 PhD Research Grant
Awarded by the Hellenic Surgical Society (Johnson & Johnson Hellas)

December 2019 PhD Research Grant
Awarded by the Hellenic Atherosclerosis Society

September 2019 Young Investigator Award in Congenital Heart Disease
Awarded by the Cardiology Society of Northern Greece

March 2019 Democracy Award
Awarded by the 2019 Model Hellenic Parliament

October 2016 Valedictorian & Summa cum Laude (M.D.)
Awarded by Aristotle University of Thessaloniki, School of Medicine

May 2016 Second place at the 1st Olympiad of Basic and Clinical Pharmacology
Awarded by the Hellenic Society for Basic and Clinical Pharmacology

FORMAL TEACHING & MENTORING EXPERIENCE

Jan 2017-Oct 2018 **Harvard Medical School, Boston, MA, USA**

June 2018-Oct 2018 Teaching Clinical Fellow in Pediatrics
Duties: *Supervised 3rd and 4th year medical students on their Pediatric Surgery and Pediatric Emergency Medicine rotations, H&P instruction, provided feedback on student notes and formal rotation evaluations*

Jan 2017-May 2018 Teaching Research Fellow in Pediatric Surgery
Duties: *Taught research methodology to Harvard medical students and residents, mentored pre-med students*

Oct 2011-July2012 **Laboratory of Descriptive Anatomy, Aristotle University**
Anatomy instructor & dissection assistant
Lab director: Prof. Konstantinos Natsis, MD, PhD
Description: *Position awarded to preclinical medical students with the highest (top 1%) performance in core Anatomy courses*

MEDICAL DEVICE DEVELOPMENT

1. Device: PLEIOFLOW RF
    - Modified IABP that increases renal perfusion during cardiac surgical procedures

- Role: Co-Investigator
- Population: Patients with Cleveland Clinic Score ≥3 or eGFR<50 ml /min/1,72 m^2
- Phase in development: First in-human trial (safety study)

BOOK CHAPTERS

1. **Mylonas K.S.**, Avgerinos D.V. ***Minimally Invasive Extracorporeal Circulation in Cardiac Surgery***. In**:** "Cardio-Pulmonary By-pass: Advances in Extracorporeal Life Support". Editors: Kaan Kırali, Joseph Coselli, Afksendiyos Kalangos, Elsevier (in-press)
2. **Mylonas K.S.**, Economopoulos K.P. ***Laparoscopic surgery for aortic vascular disease***. In**:** "Operative Endoscopic and Minimally Invasive Surgery". Editors: Daniel B. Jones, Steven D. Schwaitzberg, CRC press, ISBN-13: 9781498708302

PUBLICATIONS

Cardiovascular research

1. Tsilimigras D.I., Oikonomou E.K., Moris D., Schizas D., Economopoulos K.P., **Mylonas K.S.**

Stem Cell Therapy for Congenital Heart Disease: A Systematic Review. Circulation. 2017 Dec 12;136(24):2373-2385.

2. **Mylonas K.S.,** Sarantis P., Kapelouzou A., Karamouzis M., Iliopoulos D.C., Nikiteas N., Schizas D. ***The aortic expression pattern of mechanosensitive stem cell genes is spatially deranged by western-type diet but can be regulated with colchicine-based anti-inflammatory treatment.*** European Heart Journal. Volume 43 (s2), October 2022, ehac544.3053
3. **Mylonas K.S.,** Sarantis P., Kapelouzou A., Karamouzis M., Kapetanakis E.I., Kontzoglou K., Iliopoulos D.C., Nikiteas N., Schizas D. ***Mechanosensitive Stem Cell Genes and Klotho in Atherosclerotic Aortas: Regulating Expression Patterns using Colchicine Regimens.*** Journal of Clinical Medicine. 31 October 2022. 11(21), 6465;
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ORAL & POSTER PRESENTATIONS

International meetings

Poster presentation at the European Society of Cardiology Congress 2022 (August 2022, Barcelona, Spain)

1. **Mylonas K.S.,** Sarantis P., Kapelouzou A., Karamouzis M., Iliopoulos D.C., Nikiteas N., Schizas D. The aortic expression pattern of mechanosensitive stem cell genes is spatially deranged by western-type diet but can be regulated with colchicine-based anti-inflammatory treatment

Poster presentation at the 18th ISDE World Congress for Esophageal Diseases (September 2022, Tokyo, Japan)

2. Lidoriki I., Syllaios A., **Mylonas K.S.,** Michalinos A., Gazouli M., Karavokyros I., Liakakos T, Schizas D. Acute nutritional and functional status changes in patients undergoing esophagectomy for cancer

Poster presentation at the 2nd Olympiad in Cardiovascular Medicine -International Symposium (April 2022, Crete, Greece)

3. **Mylonas K.S.,** Tasoudis P., Pavlopoulos D., Kanakis M., Stavridis G.T., Avgerinos D.V. Aortic valve neocuspidization using the Ozaki technique: A meta-analysis of reconstructed patient-level data

Orally presented at the 5th International Forum in Pediatric and Adult Congenital Cardiology and Cardiac Surgery (April 2021, Athens, Greece)

4. **Mylonas K.S.** What's New in the Management of Neonatal Critical Aortic Stenosis: Transcatheter vs Surgical Management

Orally presented at the 2019 Meeting of the European Society for Diseases of the Esophagus (November 2019, Athens, Greece)

5. **Mylonas K.S.,** Schizas D., Hasemaki N., Mpaili E., Ntomi V., Michalinos A., Theochari N., Theochari C., Mpoura M., LiakakosT. Esophagectomy for Esophageal Cancer in an Upper GI Unit of the National and Kapodistrian University of Athens During the Period 2004-2019.

Poster presentation at the 2019 Meeting of the European Society for Diseases of the Esophagus (November 2019, Athens, Greece)

6. Davakis S., Syllaios A., Karydakis L., Hasemaki N., Skotsimara A., **Mylonas K.S.,** Tsiboukelis A., Papalampros A., Charalabopoulos A. Laparoscopic repair of a giant Morgagni's hernia presenting with respiratory distress.

Orally presented at the 13th International Gastric Cancer Congress (May 2019, Prague, Czech Republic)

7. Schizas D., Hasemaki N., **Mylonas K.S.,** Mpaili E., Ntomi V., Michalinos A., Theochari N.,

Theochari C., Mpoura M., Krivan S., Bakopoulos A., Liakakos T. Gastric and gastroesophageal junction carcinomas: An analysis of the gastrectomy databases of two University Hospitals in Greece.

Orally presented at the International Romanian Congress of Surgery (May 2014, Sinaia, Romania)

8. Sapalidis K., **Mylonas K.S.**, Anastasiadis I., Strati TM, Kesisoglou I., Kanellos I: Minimally Invasive Video-Assisted Total Thyroidectomy (mi V.A.T.T.). Our center's experience.

US meetings

Poster presentation at the 2020 American Transplant Congress (June 2020, Philadelphia, PA)

9. Ziogas I.A., Giannis D., Kapsampelis P. Giannopoulou A., **Mylonas K.S.**, Alexopoulos S.P., Tsoulfas G. Combined Heart-Liver Transplantation: A Systematic Review of the Literature

Oral presentation at the 13th Annual Conference of the International Liver Cancer Association (September 2019, Chicago, IL)

10. Ziogas I.A., Evangeliou A., **Mylonas K.S.**, Athanasiadis D., Cherouveim P., Geller D.A., Alexopoulos S.P., Tsoulfas G., Papadopoulos V. Economic Analysis of Open vs Minimally Invasive Hepatectomy: A Systematic Review and Meta-Analysis

Poster presentation at the 8th Annual Harvard Medical School Surgery Research Day (April 2019, Boston, MA)

11. Cramm S.L., Luchurst C.M., Pietrantonio N.M., **Mylonas K.S.**, Masiakos P.T., Doody D.P., Ryan D.P. Implementation of a standardized post-Nuss repair pathway using multimodal analgesia resulted in decreased in-hospital opioid use and length of stay: a single-center experience

Poster presentation at the 2018 Annual Congress of Neurological Surgeons (October 2018, Houston, TX)

12. Maragkos GA, Geropoulos G., Kechagias K. Ziogas IA, **Mylonas K.S.** Quality of life after epilepsy surgery in children: A systematic review & meta-analysis

Poster presentation at the 2018 Clinical Congress of the American College of Surgeons (October 2018, Boston, MA)

13. Ziogas I.A., Schizas D., Tsoulfas G., **Mylonas K.S**. Diffuse esophageal leiomyomatosis in pediatric patients: A Systematic Review and Evidence Quality Assessment

Poster presentation at the 7th Annual Harvard Medical School Surgery Research Day (April 2018, Boston, MA)

14. **Mylonas K.S.**, Kao C.S., Levy D., Lordello L., Dal Cin P., Masiakos PT, Oliva E. Clinical Implications of Isochromosome 12p Status in Pediatric Sacrococcygeal Teratomas

Oral presentation at the 2018 John M. Templeton Jr. Pediatric Trauma Symposium (March 2017, Philadelphia, PA)

15. Masiakos PT, **Mylonas K.S.** Managing Solid Organ Injuries? Throw Out The Grade Book!

Oral presentation at the MGH Pediatric Surgery Grand Rounds (September 2017, Boston, MA)

16. **Mylonas K.S.**, Wang G.X., Mitchell J., Sagar P., Shailam R., Kelleher C.M., Cohen A.R., Gee M.S. Risk Stratification of Non-Diagnostic Ultrasounds to Guide Management and CT

Utilization in Pediatric ED Patients Suspected of Acute Appendicitis

Oral presentation at the 2017 Harvard Trauma & Critical Care Symposium (November 2017, Boston, MA)

17. Masiakos PT, **Mylonas K.S.** Why Transfer Low and Medium Injury Severities to Pediatric Centers?

Poster presentation at the 2017 Clinical Congress of the American College of Surgeons (October 2017, San Diego, CA)

18. **Mylonas K.S.,** Ott L., Westfal M., Reinhorn M., Masiakos PT: Opioid Analgesia Requirements after Kugel Repair for Inguinal Hernia: A Call for Data-driven, Procedure-Specific Opioid-Prescribing Patterns

Oral presentation at the 2017 Surgical Advocacy Summit: Massachusetts Opioid Epidemic-The Surgeon's Response to a Public Health Crisis (October 2017, Massachusetts State House, Boston, MA)

19. **Mylonas K.S.,** Ott L., Westfal M., Reinhorn M., Masiakos PT: A Call for Data-driven, Procedure-Specific Opioid-Prescribing Patterns

Poster presentation at the 2017 MGH Clinical Research Day (October 2017, Boston, MA)

20. **Mylonas K.S.,** Ott L., Westfal M., Masiakos PT, Reinhorn M.: Near-Elimination of Opioid Use in Inguinal Herniorrhaphy Using a Systematic, Patient Centered and Procedure Specific Approach to Perioperative Pain Management

Oral presentation at the MGH Pediatric Surgery Grand Rounds (September 2017, Boston, MA)

21. **Mylonas K.S.,** Masiakos PT, Kelleher CM: Malignant pancreatic tumors in children: Histology, genetic features, and outcomes

Poster presentation at the Newton-Wellesley Hospital Research Grand Rounds (June 2017, Newton, MA)

22. **Mylonas K.S.,** Ott L., Westfal M., Masiakos PT, Reinhorn M.: Near-Elimination of Opioid Use in Inguinal Herniorrhaphy Using a Systematic, Patient Centered and Procedure Specific Approach to Perioperative Pain Management

Poster presentation at the 6th Annual Harvard Medical School Surgery Research Day (May 2017, Boston, MA)

23. **Mylonas K.S.,** Nasioudis D., Masiakos PT, Kelleher CM. Malignant Pancreatic Tumors in Children: A SEER database analysis

Oral presentation at the MGH Pediatric Surgery Grand Rounds (March 2017, Boston, MA)

24. **Mylonas K.S.,** Kelleher CM., Masiakos PT: Non-operative management of uncomplicated acute appendicitis in pediatric patients: A systematic review

Greek National meetings

Oral presentations at the 14th Panhellenic Congress of the Hellenic Society of Thoracic and Cardiovascular Surgeons (November 2022, Thessaloniki, Greece)

25. **Mylonas K.S.**, Papakonstantinou N.A., Avgerinos D.V., Mylonakis M., Frangoulis S., Kantidakis G., Stavridis G.T. HAART geometric ring annuloplasty for aortic valve repair:

the Onassis Cardiac Surgery Center Experience

26. **Mylonas K.S.,** Sarantis P., Kapelouzou A., Karamouzis M., Iliopoulos D.C., Nikiteas N., Schizas D. Developmental Genes and Klotho in Aortic Atherosclerosis: Addressing Multilevel Dysregulation using Colchicine Regimens

Invited lecture at the 14th Panhellenic Congress of the Hellenic Society of Thoracic and Cardiovascular Surgeons (November 2022, Thessaloniki, Greece)

27. **Mylonas K.S.** Statistical methodology for Cardiothoracic Surgeons

Invited lecture at the 2022 Surgical Research Seminar of the School of Medicine, National and Kapodistrian University of Athens (April 2022, Athens, Greece)

28. **Mylonas K.S.** How to write a PhD

Poster presentation at the 9thAnnual Congress of the Panhellenic Atheromatosis Society (December 2020, Athens, Greece)

29. **Mylonas K.S.,** Kapelouzou A., Spartalis M.D,, Mastrogeorgiou M., Spartalis E.D., Bakoyiannis C., Liakakos T., Schizas D., Nikiteas N., Iliopoulos D.C. Combining colchicine with clofibrate or N-acetylcysteine curtails the development of thoracic aortic atheromatosis in an experimental rabbit model

Oral presentation at the 40th Panhellenic Congress of Cardiology (October 2019, Athens, Greece)

30. Spartalis M.D, Mastrogeorgiou M., **Mylonas K.S.,** Tzatzaki E., Spartalis E.D., Nikiteas N., Iliopoulos D.C. Effect of colchicine with clofibrate or N-acetylcysteine on triglyceride levels and atheromatosis plaques in an experimental rabbit model

Oral presentations at the 12th Congress of Interventional Cardiology and Electrophysiology (September 2019, Thessaloniki, Greece)

31. **Mylonas K.S.,** Kavvouras C., Karouli P., Avramidis D., Kalangos A., Anderson D.R., Tzifa A. Rapid Pacing for Balloon Aortic Valvuloplasty Helps Prevent High-Risk Surgical Bailout Procedures During the First Year of Life
32. **Mylonas K.S.,** Tzifa A. Modified Septostomy Technique in the Setting of Transposition of Great Arteries with Aneurysmal and Intact Atrial Septum
33. **Mylonas K.S.,** Repanas T., Athanasiades D., Voulgaridou K., Sfyridis P., Bakoyiannis C., Kapelouzou A., Avgerinos D.V., Tzifa A., Kalangos A. Pacemaker Dependency after Pediatric Heart Transplant: A Systematic Review & Evidence Quality Assessment

Oral presentations at the 2019 Annual North Hellenic Congress (April 2019, Thessaloniki, Greece)

34. Ziogas I.A., Athanasiadis D., Karampet K, Spartalis E., Nikiteas N., Schizas D., Tsoulfas G., Papadopoulos V., Avgerinos D., **Mylonas K.S**. Genetic Predictors in Surgical Repair of Tetralogy of Fallot: A Systematic Review and Evidence Quality Assessment
35. Ziogas I.A., Athanasiadis D., Cherouveim P., Evangeliou A., **Mylonas K.S.,** Tsoulfas G., Papadopoulos V. Comparing Healthcare cost between Open and Laparoscopic Hepatic Surgery: A Systematic Review and Evidence Quality Assessment

Poster presentation at the 2018 Annual Panhellenic Congress of Surgery (November 2018, Athens, Greece)

36. Theochari N., Stefanopoulos A., **Mylonas K.S.,** Economopoulos K.P. Antibiotics Exposure And Inflammatory Bowel Diseases: A Systematic Literature Review

Orally presented at the 21st annual conference and 9th International forum for Medical Students and Young Physicians (May 2015, Athens, Greece):

37. **Mylonas K.,** Papavramidis T. Subtotal adrenalectomy: Current indications, post-operative results and future prospects.

Presented at the 1stPanhellenic Congress on Continuing Education in Internal Medicine with International participation (February 2015, Thessaloniki, Greece)

38. **Mylonas K.,** Deligiannis G., Papadatos S., Zisis Ch, Mylonas S: Case report: Fulminant AIDS in a chronic carrier with the first manifestation being hemoptysis due to pancytopenia (Oral presentation)
39. Papadatos S., Deligiannis G., Gatsa E., **Mylonas K.,** Mylonas S.: Case report: Acute chest syndrome as initial presentation in a patient with Thrombotic Thrombocytopenic purpura. (Oral presentation)
40. Deligiannis G., Papadatos S., **Mylonas K.** Zisis Ch., Mylonas S.: Case report: Acute renal injury in Weil's disease. The importance of medical awareness (Poster)
41. Zisis Ch., Deligiannis G., Papadatos S., **Mylonas K.,** Mylonas S.: Uncommon clinical and radiographic findings of pulmonary Brucellosis: acaseseries (Poster)

Presented at the 20th annual conference and 8th International forum for Medical Students and Young Physicians (May 2014, Thessaloniki, Greece):

42. **Mylonas K.,** Dimitriades C.: Non–palpable breast lesions: Current Diagnosis and Treatment (oral presentation)
43. **Mylonas K.,** Myronidou-Tzouveleki M: Trastuzumab (Herceptin ®). Current views on target therapy(E-poster)
44. **Mylonas K.,** Iakovis N, Baili G, Zisis Ch., Mylonas S: Case report: Diagnosis of pernicious anemia with vitiligo as presenting manifestation (E-poster)
45. **Mylonas K.,** Iakovis N, Baili G, Zisis Ch, Mylonas S: Cross-sectional study of infectious mononucleosis cases treated at the General Hospital of the Municipality of Trikala in the period 2009-2013(E-poster)

Orally presented at the 2013 Annual Oncology Meeting for Medical Students (November 2013, Heraklion, Greece)

46. **Mylonas K.,** Revithis A., Karachaliou G., Moustaka G.,Tsika A., Manolakou S.: Nrf2/ Keap 1 path: impact on the prognosis of Small Cell Lung Cancer.

Orally presented at the 26th Annual Conference of the North Hellenic Diabetes Association (November 2012, Thessaloniki, Greece).

47. **Mylonas K.,** Mylonas S., Gkaleas T: Correlation study between ion/water interchangeability of alluvial soils and diabetes mellitus type 2 in inhabitants of the Municipality of Trikala.

Orally presented at the 18th annual conference and 6th International forum for Medical Students and Young Physicians (May 2012, Athens, Greece)

48. **Mylonas K.,** Pantelidis P, Karadaglis P, Imprialos K. Myronidou-Tzouveleki M: Pharmaceutical treatment of the HIV infection: Current paradigm and review of ongoing clinical trials
49. **Mylonas K.,** Leptokaridou E., Natsis K: Piriformis and Obturator nerve tunnel syndromes: Results of cadaver studies and literature review

EDITORIAL APPOINTMENTS

June 2022-ongoing **Journal of Clinical Medicine**
Role: Associate Editor
Special Issue: New Perspectives in Cardiothoracic Surgery
Journal Impact factor: 4.97
Co-Editor: Dimitrios Angouras, MD, PhD, Professor of Cardiothoracic Surgery, National and Kapodistrian University of Athens

JOURNAL REVIEWER APPOINTMENTS

Reviewer at:
-Cells
-PLOS Medicine
-European Journal of Vascular and Endovascular Surgery
-Scientific Reports
-Oncotarget
-The Journal of Pediatrics
-Archives of Disease in Childhood
-Congenital Heart Disease
-World Journal for Pediatric and Congenital Heart Surgery
-Cardiology in the Young
-World Journal of Surgery
-World Journal of Gastroenterology
-Pediatric Blood & Cancer
-BMJ Case Reports
-The Journal of Pain Research
-Journal of Investigative Surgery
-World Journal of Surgical Oncology
-World Journal of Clinical Oncology
-International Journal of Surgery

CONTINUOUS EDUCATION

April 2021	**Cardiovascular Anastomosis Skills Workshop** Ethicon (Johnson & Johnson) Proctor: Professor Paul T. Sergeant, MD, PhD
Aug 2018	**Pediatric Advanced Life Support (PALS)** American Heart Association
Aug 2018	**Basic Life Support (BLS)** American Heart Association
Nov 2017	**HCUP Data Use Agreement Training** Agency for Healthcare Research and Quality
Jul-Sep 2017	**Introduction to Mixed Methods Research** Harvard Clinical and Translational Science Center
June 2017	**ECFMG certification** Educational Committee for Foreign Medical Graduates
March-April 2017	**Applied Methods in Designing and Conducting Health Surveys** Mongan Institute Health Policy Center-Massachusetts General Hospital
Feb-March 2017	**Advanced Training in Statistical Coding with R** Harvard Clinical and Translational Science Center
Jan 2017	**Collaborative Institutional Training Initiative (CITI program)** Biomedical Research Investigators and Key Personnel
September 2015	**Infection Control Mandated Training** New York State Education Department Code: 653TOK, Grade: 100%

LEADERSHIP EXPERIENCE& VOLUNTEER WORK

Nov 2018-Mar 2019	**Hellenic Parliament** Member of the 2019 Model Hellenic Parliament Committee: Economics *Received the prestigious Democracy Award*

July 2014-Dec 2018 **Society of Junior Doctors (SJD), Athens, Greece**
Jan 2016-Dec 2018 *Chair, Surgery Working Group*
Jan 2018-Dec 2018 *Member of the Audit Committee*
Nov 2017-Dec 2018 *Founder and Director of the Hellenic Honors Medical Society*
Jan 2017-Dec 2017 *Chairman of the Board*
Jan 2016-Dec 2017 *Member of the Board*

Dec 2015 **NIH Clinical Center-Aristotle University of Thessaloniki affiliation**
Founder

August-October 2015 **Internal Medicine sub-internship, AHEPA University Hospital**
Administrative Sub-intern and Student representative

PROFESSIONAL MEMBERSHIPS

Member of:

- The Society of Thoracic Surgeons
- American College of Surgeons
- American Heart Association
- European Society of Cardiology
- American Medical Association
- Pediatric Congenital Heart Association
- New England Hellenic Medical and Dental Society
- Athens Medical Association
- Hellenic Society of Cardiovascular and Thoracic Surgeons
- Hellenic Surgical Society
- Hellenic Society of Atherosclerosis

LANGUAGES

April 2015 **TOEFL iBT**
Score: 117/120
Reading: 30/30, Writing: 30/30, Speaking: 29/30, Listening: 28/30
Registration Number: 0000000024536516

COMPUTER SKILLS

- **STATA:** Full working proficiency
- **REDCap programming**: Full working proficiency

Abbreviations

A
APC - antigen-presenting cell
ADP - adenosine diphosphate
ACS - acute coronary syndrome
ApoE – Apolipoprotein E
ApoB- Apolipoprotein B
ABCA1 - ATP-binding cassette transporter A1
AJ - adherens junction
ARNT - aryl hydrocarbon receptor nuclear translocator
ACE - Angiotensin-converting enzyme
ALP- alkaline phosphatase
AST - aspartate aminotransferase
ALT - alanine aminotransferase
ANOVA - analysis of variance

B
BMP - bone morphogenetic protein
BMPR1 – BMP receptor type I
BMPR2– BMP receptor type II
BCA – branchiocephalic artery
bp – base pair

C
CVD - cardiovascular disease
CAD - coronary artery disease
CVA - cerebrovascular accidents
CD - cluster differentiation
CXCL4 - chemokine (C-X-C motif) ligand 4
CHIP - clonal hematopoiesis of indeterminate potential
CRP – C reactive protein
CANTOS trial - Canakinumab Anti-Inflammatory Thrombosis Outcomes trial
CI – confidence interval
CKD - chronic kidney disease
COLCOT trial- colchicine cardiovascular outcomes trial
COX – cyclooxygenase
CIRT - Cardiovascular Inflammation Reduction Trial
CREB - Cyclic adenosine monophosphate Response Element Binding
CDKN1A - Cyclin Dependent Kinase Inhibitor 1A
CT – Computed tomography
Col- colchicine
CD- cholesterol diet
Cx40 – Connexin 40

D
DNA - deoxyribonucleic acid
DLL1 – delta ligand 1
DNMT1 - DNA methyltransferase 1
DGAT1 - diacylglycerol acyltransferase 1

E
ER - endothelial reticulum
ENT - equilibrative nucleoside transporter
ESC – embryonic stem cell
ERK - Extracellular Signal-Regulated Kinase
EGF- Epidermal growth factor

F
FDG – fluorodeoxyglucose
Fen - fenofibrate

G
GRB2 - Growth Factor Receptor-Bound Protein-2)
GAPDH - glyceraldehyde 3-phosphate dehydrogenase

H
hsCRP – high sensitivity C reactive protein
HMG-CoA- 3-hydroxy-3 -methylglutaryl-Coenzyme A
HR – hazard ratio
HDL - high-density lipoprotein
HIF1α - hypoxia- inducible factor 1a
HMG - high-mobility-group
HPH - HIF prolyl hydroxylase
HRE - hypoxia response element
HUVEC - human umbilical vein endothelial cell

I
IL – interleukin
ICAM 1 - intercellular adhesion molecule-1
IFN – interferon
iPSC - induced pluripotent stem cell
IEL - internal elastic lamina

J
JUPITER trial- Justification for the Use of Statins in Prevention: an Intervention Trial Evaluating Rosuvastatin
JAK - Janus kinase
JAG1 - Jagged-1
JAG2 - Jagged-2

K
KLF - Krüppel-like factor

L
LDL - low-density lipoproteins

LOX1 - lectin-like oxidized LDL receptor 1
LIF - leukemia inhibitory factor
Lp-PLA2 - Lipoprotein-associated phospholipase A2
LoDoCo - low dose colchicine
LNR - LIN-12/notch repeat

M
M-CSF - macrophage-colony-stimulating factor
MIP - macrophage inflammatory protein
MI - myocardial infarction
MAPK - mitogen-activated protein kinase
MMP - matrix metalloproteinase
MPO – myeloperoxidase
MACE - major adverse cardiovascular event
mRNA – messenger ribonucleic acid
MRI – Magnetic resonance imaging

N
NF kB - nuclear factor kB
NO - nitric oxide
NOS - nitric oxide synthase
NET - neutrophil extracellular trap
NLRP3 - NLR family pyrin domain containing 3
NAC – N acetylcysteine
NLS - nuclear localization signal
NADPH - nicotinamide adenine dinucleotide phosphate
NICD - Notch intracellular domain
NEXT - Notch extracellular truncation
Nrf2 - Nuclear factor erythroid 2-related factor 2
NWI - normalized wall index

O
OCT4 - Octamer-binding transcription factor 4

P
PAMP - pathogen-associated molecular patterns
PRR - pattern recognition receptor
PLATO trial- platelet inhibition and patient outcomes trial
PPAR - peroxisome proliferator activated receptor
PSBP - Pluripotential cell-specific SOX element-Binding Protein
PHD - prolyl hydroxylase
PFKFB3 - phosphofructo-2-kinase/fructose-2,6-biphosphatase 3
PET - Positron emission tomography

Q
qRT-PCR - quantitative real-time polymerase chain reaction

R
ROS - reactive oxygen species

RANTES - Regulated upon Activation, Normal T Cell Expressed and Presumably Secreted
RNA - ribonucleic acid
RANKL - receptor activator of nuclear factor kappa-B ligand
RIG - retinoid acid-inducible gene

S
SMC - smooth muscle cell
STAT - Signal Transducers and Activators of Transcription
SRY - sex-determining region Y
SOX - Sry-related HMG box
SD – standard deviation

T
TNF - tumor necrosis factor
TLR - toll-like receptor
TREG - regulatory T-lymphocytes
TET 2 - Tet methylcytosine dioxygenase 2
TGF β - transforming growth factor- β
TG – triglycerides
TWIST1 - twist related protein 1
TAD - transactivation domain
TWEAK - TNF-like weak inducer of apoptosis
TC – total cholesterol

U
USA - United States of America

V
VCAM 1 - vascular cell adhesion molecule 1
VE cadherin - vascular endothelial cadherin
VEGF - vascular endothelial growth factor
VSMC – vascular smooth muscle cell

W
-

X
-

Y
YAP - Hippo–Yes- associated protein

Z
-

Abstract

Purpose: Inflammatory dysregulation of mechanosensitive genes is central to atherogenesis. In the present animal model, we utilized colchicine regimens to curtail *de novo* aortic atherogenesis in white New Zealand rabbits. We also explored the effect of colchicine-based treatment on a variety of atheroprotective (*KLF4, Klotho, HOXA5, NOTCH1, OCT4*) and proatherogenic (*HIF1a, SOX2, BMP4, NANOG*) genes.

Methods: The control (n=6) and group A (n=6) received standard and cholesterol-enriched chow, respectively. Groups B (n=8) and C (n=8) were fed hypercholesterolemic diet and were treated with colchicine plus fenofibrate or N-acetylcysteine (NAC), respectively.

Results: Group A developed significantly greater thoracic and abdominal aortic atherosclerosis compared to groups B ($p<0.001$) and C ($p<0.001$). Combining colchicine with NAC resulted in stronger atheroprotection both in the thoracic and the abdominal aorta. Group A exhibited significantly greater thoracic *KLF4* expression compared to groups B (MD: 4.94, 95% CI: 1.11-8.77) and C (MD: 9.94, 95% CI: 6.11-13.77). Combining colchicine with NAC (MD: 5.00, 95% CI: 1.45-8.54) led to a more robust reduction in thoracic *KLF4* expression. No difference was observed in terms of abdominal *KLF4* expression between controls and group A, whereas colchicine-based treatments were equally effective in upregulating it. In group A thoracic aortas, *Klotho* was downregulated compared to controls (95% CI: 1.82-15.76). Both colchicine regimens upregulated *Klotho* back to baseline levels ($p<0.001$). Colchicine/fenofibrate also significantly upregulated thoracic *NOTCH1* compared to controls (95% CI: -8.09 to -0.48). Colchicine/NAC significantly reduced thoracic *NANOG* expression compared to hyperlipidemic diet alone (95% CI: 0.37-8.29). In the abdominal aorta, hypercholesterolemic diet resulted in significant downregulation of *HOXA5* (95% CI: 0.03-2.74) which was reversed with colchicine/NAC back to baseline (95% CI: -1.19 to 1.51). Colchicine/fenofibrate downregulated *HIF1a* compared to controls (95% CI: 0.83-6.44). No significant differences were noted in terms of *BMP4*, *SOX2*, and *OCT4*.

Conclusions: The expression pattern of aortic stem-cell genes was spatially influenced by Western-type diet and could be modified using colchicine regimens. Hyperlipidemic diet drove de novo thoracic and abdominal aortic atherogenesis by downregulating *α-Klotho* and *HOXA5*, respectively. Both colchicine regimens halted thoracic aortic atheromatosis by upregulating *α-Klotho*. In the thoracic aorta, combining colchicine with fenofibrate also increased *NOTCH1*, while the addition of NAC reduced *NANOG*. In the abdominal aorta, combining colchicine with fenofibrate reduced *HIF1a*, whereas the addition of NAC upregulated *HOXA5*.

Περίληψη

Σκοπός: Η απορρύθμιση των μηχανοευαίσθητων βλαστοκυτταρικών γονιδίων είναι κεντρικής σημασίας για την αθηρογένεση. Στο παρόν μοντέλο, χρησιμοποιήσαμε σχήματα κολχικίνης για να περιορίσουμε την *de novo* αορτική αθηρογένεση σε λευκά κουνέλια Νέας Ζηλανδίας. Εξερευνήσαμε επίσης την επίδραση της θεραπείας σε αθηροπροστατευτικά (*KLF4, Klotho, HOXA5, NOTCH1, OCT4*) και προαθηρογόνα (*HIF1a, SOX2, BMP4, NANOG*) γονίδια.

Μέθοδοι: Οι μάρτυρες (n=6) και η ομάδα Α (n=6) έλαβαν κλασική τροφή και εμπλουτισμένη σε χοληστερόλη, αντίστοιχα. Οι ομάδες Β (n=8) και C (n=8) τράφηκαν με υπερχοληστερολαιμική δίαιτα και έλαβαν θεραπεία με κολχικίνη συν φενοφιβράτη ή N-ακετυλοκυστεΐνη (NAC), αντίστοιχα.

Αποτελέσματα: Η ομάδα Α ανέπτυξε εκτενέστερη αθηροσκλήρυνση θωρακικής και κοιλιακής αορτής σε σύγκριση με τις ομάδες Β ($p<0.001$) και C ($p<0.001$). Ο συνδυασμός κολχικίνης με NAC προσέφερε ισχυρότερη αθηροπροστασία τόσο στη θωρακική όσο και στην κοιλιακή αορτή. Η ομάδα Α εμφάνισε σημαντικά υψηλότερη έκφραση θωρακικού *KLF4* συγκριτικά με τις ομάδες Β (MD: 4.94, 95% CI: 1.11-8.77) και C (MD: 9.94, 95% CI: 6.11-13.77). Ο συνδυασμός κολχικίνης με NAC (MD: 5.00, 95% CI: 1.45-8.54) οδήγησε σε εντονότερη μείωση του θωρακικού *KLF4*. Εντούτοις, δεν υπήρχε διαφορά στο κοιλιακό *KLF4* μεταξύ των μαρτύρων και της ομάδας Α. Οι θεραπείες κολχικίνης ενίσχυσαν εξίσου αποτελεσματικά την έκφραση του παραπάνω γονιδίου. Στη θωρακική αορτή της ομάδας Α, η *Klotho* ήταν μειωμένη συγκριτικά με τους μάρτυρες (95% CI: 1.82-15.76). Και τα δύο σχήματα κολχικίνης επανέφεραν τη Klotho στα αρχικά επίπεδα ($p<0.001$). Η κολχικίνη/φενοφιβράτη αύξησε σημαντικά το θωρακικό *NOTCH1* συγκριτικά με τους μάρτυρες (95% CI: -8.09 έως -0.48). Η κολχικίνη/NAC μείωσε την έκφραση θωρακικού *NANOG* σε σύγκριση με μόνο την υπερλιπιδαιμική δίαιτα (95% CI: 0.37-8.29). Στην κοιλιακή αορτή, η υπερχοληστερολαιμική δίαιτα οδήγησε σε σημαντική μείωση του *HOXA5* (95% CI: 0.03-2.74) η οποία αναστράφηκε με κολχικίνη/NAC (95% CI: -1.19 έως 1.51). Η κολχικίνη/φενοφιβράτη μείωσε το *HIF1a* σε σύγκριση με τους μάρτυρες (95% CI: 0.83-6.44). Δεν σημειώθηκαν σημαντικές διαφορές όσον αφορά τα *BMP4*, *SOX2* και *OCT4*.

Συμπεράσματα: Η έκφραση των αορτικών βλαστοκυτταρικών γονιδίων επηρεάζεται χωρικά από τη δίαιτα δυτικού τύπου αλλά μπορεί να ρυθμιστεί με σχήματα κολχικίνης. Η υπερλιπιδαιμική δίαιτα προκάλεσε αθηρογένεση της θωρακικής και της κοιλιακής αορτής κυρίως λόγω της μείωσης των *α-Klotho* και *HOXA5*, αντίστοιχα. Και τα δύο σχήματα κολχικίνης περιόρισαν την αθηρωμάτωση της θωρακικής αορτής αυξάνοντας την *α-Klotho*. Στη θωρακική αορτή, ο συνδυασμός κολχικίνης με φενοφιβράτη αύξησε το *NOTCH1*, ενώ η προσθήκη NAC μείωσε το *NANOG*. Από την άλλη, στην κοιλιακή αορτή, ο συνδυασμός κολχικίνης με φενοφιβράτη μείωσε το *HIF1α*, ενώ η προσθήκη NAC ενίσχυσε το *HOXA5*.

Theoretical Framework

CHAPTER 1

Fundamentals of atheromatosis

1.1 Definition of atheromatosis

Atheromatosis or atherosclerosis is a term used to describe the accumulation of fatty and/or fibrous material in the innermost layer of arteries (aka the intima). The term atherosclerosis stems from the Greek word for “gruel” or “porridge”, reflecting the appearance of the lipid material found in the core of the typical atherosclerotic plaque (or atheroma). With time, the atherosclerotic plaque can become more fibrous and accumulate calcium mineral. Advanced atherosclerotic plaques can encroach upon the arterial lumen, impeding blood flow, and eventually leading to tissue ischemia. Atheromata that do not produce a flow-limiting obstruction can disrupt blood flow and provoke the formation of a thrombus causing rapid lumen occlusion thereby leading to acute ischemic events.[1]

1.2 Clinical manifestations of atheromatosis

Atherosclerotic plaques usually develop at sites of hemodynamic shear stress, such as curvatures, branches, and bifurcations of large arteries where disturbed blood flow prevails.[2, 3] This is typically characterized by laminar flow separation, transient flow reversals, and low shear

forces that predispose to atherogenesis. On the other hand, pulsatile unidirectional laminar undisturbed flow produces high shear forces and usually prevails in atheroresistant regions.[4]

Atheromatosis is a diffuse, slowly progressing disease that can affect several arterial beds. Given the indolent nature of the disease, many patients remain asymptomatic for decades. The clinical presentation of atherosclerosis can be acute or chronic. This varies substantially, depending on the vascular territory involved. **Figure G1.1** provides a synopsis of cardinal atherosclerotic manifestations.

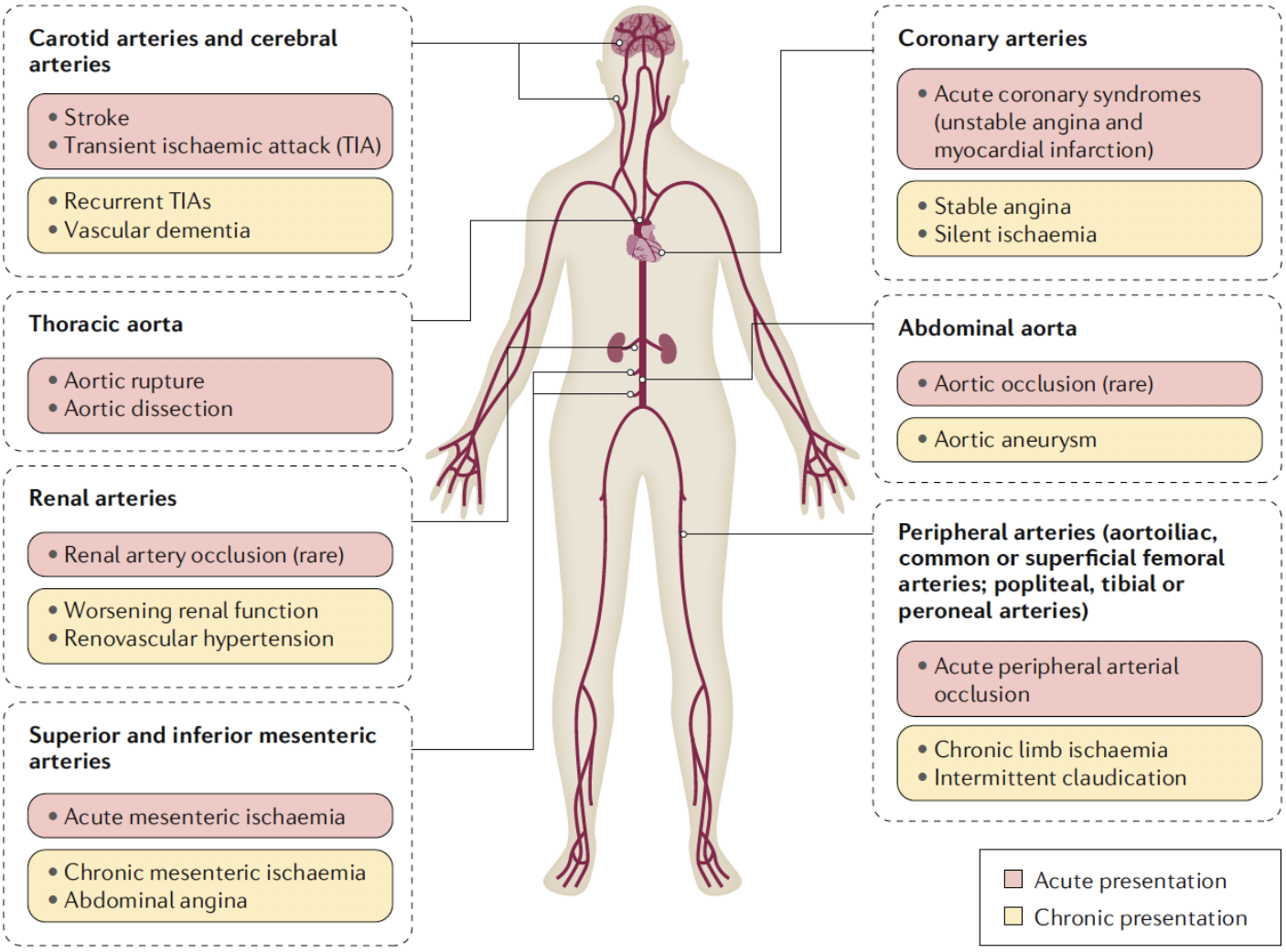


Figure G1.1. Clinical manifestations of atherosclerosis *(adapted from Libby P., et al., Atherosclerosis. Nat Rev Dis Primers, 2019. 5(1): p. 56)*

1.3 Epidemiology of atheromatosis

As previously shown, atherosclerotic cardiovascular disease (CVD), includes, among other entities, coronary artery disease (CAD), hypertension, and cerebrovascular accidents (CVAs), which collectively comprise the number one cause of death globally. Over 17 million people died from CVD in 2015, representing 31% of all global deaths. Of these, an estimated 7.4 million occurred due to coronary heart disease and 6.7 million due to stroke.[5] The direct cost of treating CVD in the United States of America (USA) currently exceeds $300 US billion per year, and predictions put both direct and indirect costs to almost a trillion US dollars by 2030.[6]

Although ischemic heart disease remains the leading cause of premature adult mortality worldwide, advances in medical, interventional, and surgical management have led to striking declines in cardiovascular mortality both in men and in women. Yet, these improvements do not seem to apply evenly across all populations. Globally, over 75% of deaths from CVD occur in low income and middle-income countries.[1, 5] In these countries, individuals with CVD have limited access to effective and equitable health-care services. This limitation can delay CVD detection until late in course of the disease thus increasing premature mortality from CVD.[7]

Patient age, race, sex, and genetic predisposition can all predispose to the development of atheromatosis - but are non-modifiable. On the other hand, tobacco use, increased salt intake, obesity, physical inactivity, hyperlipidemia, hypertension, diabetes mellitus, and high alcohol use can be prevented thereby decreasing atherosclerotic stimuli. Being proactive regarding preventive measures is crucial considering the fact that exposure to risk factors has a cumulative effect throughout life.

CHAPTER 2

Inflammation in the development of atheromatosis

2.1 Retention and oxidization of lipoproteins

Both the innate and the adaptive arms of the immune system partake in various steps of atherogenesis (**Figure G2.1**). The first step in the development of atherosclerosis entails the accumulation of low-density lipoproteins (LDL) in the subendothelial space by adhering to proteoglycan-rich extracellular matrix proteins.[8] LDL can accumulate as a result of alterations in endothelial permeability and paracellular transport among leaky cells or, as more recently described, through active receptor-mediated transcytosis across the cell membrane by transporters like SR-BI and Alk1.[9, 10] This body of literature has explained how the endothelium enables the accumulation of large particles like LDL.

Once in the sub-intimal space, LDL can undergo modification, aggregates, and becomes oxidized. The aggregation of LDL results in large complexes ranging in size from 100 nm to 1.0 mm which can undergo pinocytosis or phagocytosis by indigenous immune cells. Reactive oxygen species (ROS) and enzymes like lipoxygenases and myeloperoxidases modify both the phospholipid and protein components of LDL particles, rendering them substrates for scavenger receptor mediated uptake. Scavenger receptors present them to innate immune phagocytes and antigen-presenting cells (APCs) which have evolved to recognize microbial and “non-self”

epitopes. Unlike native LDL uptake through the LDL receptor, scavenger receptor-mediated uptake is not subject to feedback inhibition by intracellular sterol levels. Therefore, phagocytosis and/or receptor mediated uptake can continue unrestricted as long as modified LDL is present in the extracellular milieu.[11]

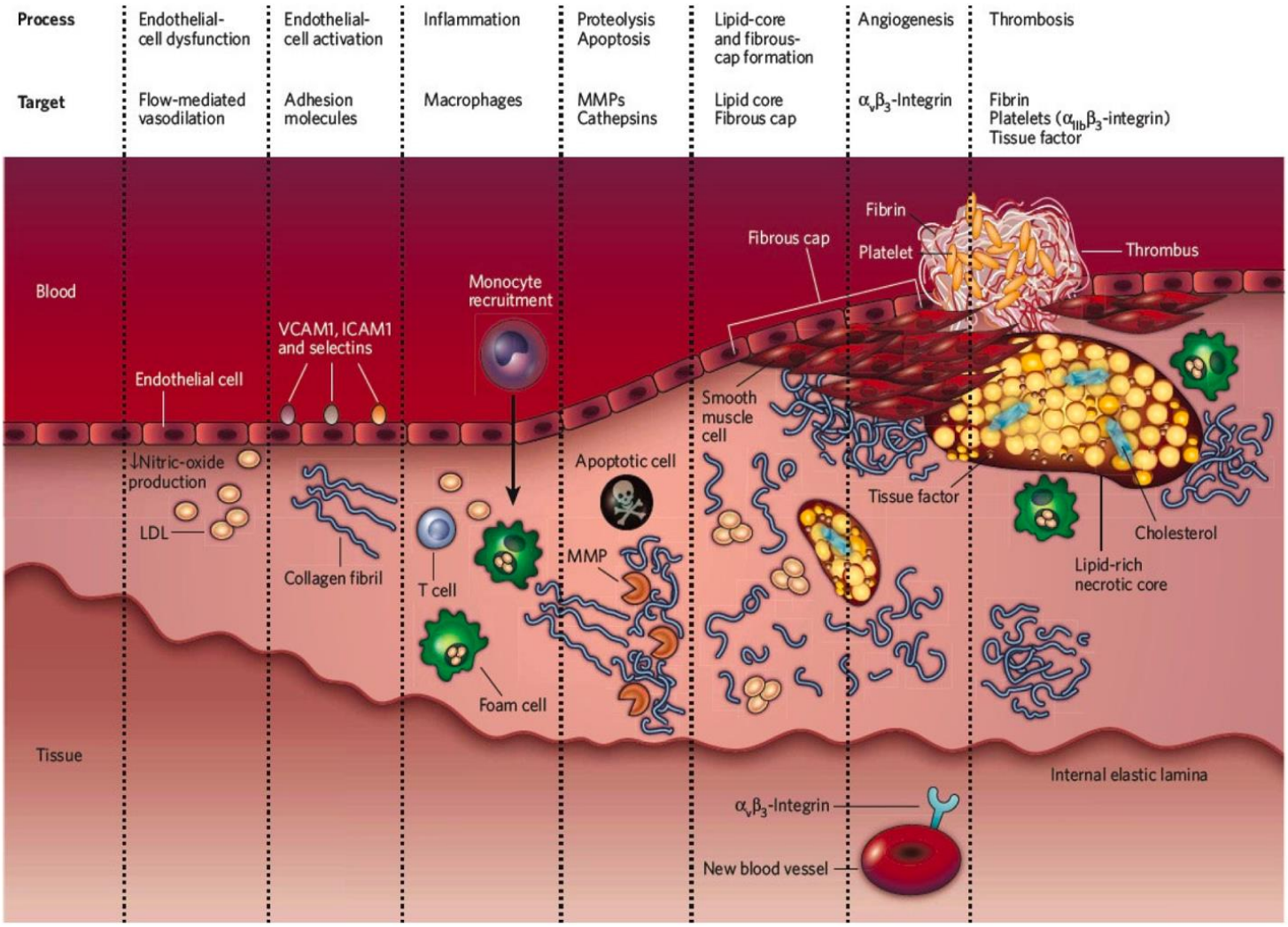


Figure G2.1. Inflammatory phenomena during atherogenesis *(adapted from Sanz J. et al. Imaging of atherosclerotic cardiovascular disease. Nature. 2008 Feb 21;451(7181):953-7.)*

2.2 Recruitment of monocytes and their differentiation into foam cells

Several noxious stimuli (such as smoking, hypertension, dyslipidemia etc.) may cause endothelial injury. Normally, the endothelium maintains a delicate balance of vasodilation, vasoconstriction and pro- and anti-coagulant activity.[12] Proinflammatory cytokines, such as tumor necrosis factor-alpha (TNF-a), interleukin 1 (IL-1), and macrophage-colony-stimulating factor (M-CSF), increase the binding of LDL to the endothelium and to vascular smooth cells whilst increasing the transcription of the LDL-receptor gene.

In the presence of noxious stimuli, the endothelium responds by upregulating nuclear factor kB (NF-kB) and via releasing a constellation of mediators that enhance leukocyte adhesion such as selectins, integrins, members of the immunoglobulin superfamily of proteins (i.e. vascular cell adhesion molecule 1 [VCAM-1] and intercellular adhesion molecule-1 [ICAM-1]), as well as endothelin, angiotensin II, and various pro-coagulant factors.[13] The role of different adhesion molecules is summarized in **Table G2.1**.

Rolling leukocytes adhere onto the endothelium and penetrate beneath the endothelial layer to reach the subintimal space. Modified lipoproteins are first absorbed by tissue-resident dendritic cells and macrophages in the arterial intima. Additionally, non-classical “patrolling” monocytes can engulf oxidized LDL via scavenger receptor cluster differentiation 36 (CD36) at primordial stages of atherogenesis.

Immune cells further induce the expression of endothelial adhesion molecules to recruit bone marrow derived monocytes into the intima.[14] As monocytes enter the subendothelial space, they differentiate into macrophages and engulf modified LDL. Excess cholesterol is esterified for storage in lipid droplets, giving macrophages their textbook foam-like appearance.

Adhesion Molecules	Cells Expressing the Molecule	Cluster of Differentiation (CD) Marker	Role
Selectins			
P-selectin	Platelets, endothelial cells	CD62P	Tethering, rolling
E-selectin	Endothelial cells	CD62E	Tethering, rolling
L-selectin	Leukocytes (neutrophils, monocytes, subsets of T cells)	CD62L	Tethering, rolling
Integrins			
LFA-1	Leukocytes (neutrophils, monocytes, subsets of T cells)	CD11a/CD18	Tight adhesion
Mac-1, CR3	Neutrophils, monocytes	CD11b/CD18	Tight adhesion
VLA-4 (very late antigen-4)	Lymphocytes, monocytes, eosinophils	CD49d/CD29	Tethering, Rolling, Tight adhesion
Immunoglobulin superfamily			
ICAM-1	Endothelial cells	CD54	Endothelial ligand for LFA-1, Mac-1, CR3. Important for tight adhesion
ICAM-2	Endothelial cells	CD102	Endothelial ligand for LFA-1. Important for tight adhesion
VCAM-1	Endothelial cells	CD106	Endothelial ligand for VLA-4. Important for tethering, rolling and tight, adhesion
PECAM-1	Leukocytes, endothelial cells	CD31	Diapedesis

Table G2.1. Key molecules involved in leukocyte-endothelial interactions *(adapted from Pant S. et al. Inflammation and atherosclerosis—revisited. J Cardiovasc Pharmacol Ther. 2014 Mar;19(2):170-8.)*

Foam cells not only induce cytokine and chemokine production but also recruit circulating immune cells, thereby setting off an inflammatory response. The activation of scavenger receptors (particularly CD36) by modified cholesterol engages innate immune responses downstream of the toll-like receptor (TLR) pathway.[15] Importantly, cholesterol crystals induce the activation of the inflammasome in the cytoplasm of the macrophages in the arterial intima. The inflammasome is a protein complex that senses exogenous danger to signal and cleave prointerleukin-1b (IL-1b) and IL-18 which are then secreted as activated cytokines.[16] IL-1a is also secreted in response to scavenger receptor activation by modified cholesterol and has been postulated to play a more

potent role in atherogenesis than IL-1b.[17] In the extracellular space, IL-1b, IL-1a and IL-18 interact with their cognate receptors and cause the release of ROS, matrix degrading enzymes, as well as the activation and proliferation of T-cells and the further production of cytokines. It should be emphasized that although certain T-cells, such as T-helper-1 (Th1) cells, play a pro-atherogenic role, others have been shown to limit atherosclerosis progression.[18] These include, Th2 cells which secrete IL-10, regulatory T-lymphocytes (TREGs) which secrete IL-10 and TGF-beta, and T-helper 17 cells which secrete IL-17.[19]

2.3 The role of platelets in pro-atherogenic inflammation

Although historically considered to have minimal impact on atherogenesis, it is now proven that platelets play a crucial role not only in acute ischemic events but also in perpetuating chronic inflammatory phenomena. From a biochemistry standpoint, platelets secrete several vasoactive chemokines and cytokines (e.g. CD40L, thrombospondin, platelet-activating factor), chemokine (C-C motif) ligand 5 (CCL5-also known as RANTES [Regulated upon Activation, Normal T Cell Expressed and Presumably Secreted]), extractable nuclear antigen ENA-78, macrophage inflammatory protein (MIP), and chemokine (C-X-C motif) ligand 4 (CXCL4) all of which have strong autocrine and paracrine effects.[20]

Activated platelets attach to leukocytes, resulting in the formation of platelet–leukocyte aggregates.[21] Thromboxane A2, a potent pro-aggregatory eicosanoid released from activated platelets, is also a proinflammatory stimulus. Furthermore, free cholesterol and oxidized LDL are potent proinflammatory stimuli triggering the recruitment of more macrophages. Oxidized LDL

itself stimulates platelet aggregation in response to adenosine diphosphate (ADP) and thrombin - most likely by activation of lectin-like oxidized LDL receptor 1 (LOX-1) on platelets.[22]

2.4 The role of vasomotor dysfunction in pro-atherogenic inflammation

Healthy endothelium produces nitric oxide (NO) from L-arginine through the enzyme NO synthase. Nitric oxide acts as a local vasodilator by increasing smooth muscle cyclic guanosine monophosphate levels. It also inhibits leukocyte adhesion and activation, platelet aggregation, and smooth muscle cell (SMC) proliferation. In the presence of atherosclerosis risk factors, the vasodilatory properties of the endothelium are attenuated. Paradoxical vasoconstriction may also be seen in large vessels as well as in the microcirculation (even in the absence of structural abnormalities in the vessel wall).

These aberrant vasomotor responses have been attributed to reduced bioavailability of constitutive NO as a result of rapid inactivation in the presence of ROS and asymmetric dimethylarginine (an inhibitor of NO production).[23] In the same context, activated leukocytes can also elicit a vasoconstrictor response by releasing leukotrienes C4 and D4 either alone or in conjunction with the secretion of thromboxane A2 from activated platelets.

2.5 The role of vascular smooth muscle cells in pro-atherogenic inflammation

SMCs possess remarkable phenotypic plasticity that enables rapid adaptation to fluctuating environmental stimuli, including drivers of atherosclerosis. Indeed, during the development of an

atherosclerotic plaque, certain SMCs can transition from a primarily contractile, nonproliferating phenotype to a proliferating, migratory and matrix-secreting state that populates the arterial intima. Furthermore, such SMCs engulf modified lipids and adopt a “macrophage-like” phenotype. These cells express macrophage markers on their surface and develop phagocytic activity.[24] Recent data suggest that up to 50% of the cells in an atherosclerotic plaque that appear to be macrophages may be derived from a SMC lineage.[25] Nevertheless, compared to their immune-derived counterparts, macrophage-descended SMCs are not as efficient in phagocytosis nor do they express abundant levels of cholesterol export machinery.

2.6 The role of cell death in pro-atherogenic inflammation

Macrophages suffer endothelial reticulum (ER) stress and undergo either apoptosis or necroptosis in response to lipid engorgement. Unless esterified, cholesterol, is highly toxic to cells and triggers the unfolded protein response in the ER. ER stress stimulates the production of CCAAT enhancer-binding protein homologous protein and initiates the release of Ca2þ from the ER. Subsequently, cytochrome C is released from the mitochondria and caspase-dependent apoptosis is activated. Apoptosis in the initial stages of lesion development is protective by reducing lesion cellularity and by eliminating cholesterol-engorged cells.[26]

This benefit depends, however, on the efficient removal of apoptotic macrophages from the lesion by efferocytosis, aka the phagocytosis of dead and dying cells. Efferocytosis in lesions is thought to occur primarily through the cell surface receptor MerTK on macrophages (although other receptors may also play a role). When MerTK activity is impaired, the clearance of dead foam cells is reduced, and apoptotic debris begins to accumulate.[27] This can also lead to

secondary necrosis, wherein an apoptotic cell that is not efficiently cleared undergoes necrotic rupture. Efferocytosis in advanced lesions might also be compromised due to the relatively limited capacity of SMC-derived foam cells to perform phagocytosis leading to the accumulation of cellular debris within the plaque.

Necroptosis is another form of programmed cell death that is activated in macrophages after prolonged exposure to oxidized lipoproteins.[28] Unlike apoptosis, necroptosis triggers an inflammatory response by the unregulated release of intracellular contents which serve as activation signals for the innate immune system. It is thought that necroptosis may be active at later stages of lesion development, particularly in humans, where the presence of dead cells with a necrotic morphology dominates over those with apoptotic characteristics. Efferocytosis of necroptotic cells is impaired, which also contributes to the expansion of the necrotic core.[29] Overall, the combination of the aforementioned cell death pathways with defective clearance of cellular debris promotes lesion necrosis, exacerbates inflammation, and ultimately contributes to the instability of advanced atherosclerotic plaques.

2.7 The role of derailed hematopoiesis in pro-atherogenic inflammation

Clonal hematopoiesis of indeterminate potential (CHIP) is common among older individuals. CHIP is an expanded somatic blood-cell clone seen in people without other hematologic abnormalities. Preliminary data suggested an association between CHIP and atherosclerotic cardiovascular disease.[30] A subsequent Harvard-led study, confirmed this observation demonstrating a two-fold risk increment in myocardial infarction (MI) and stroke in CHIP-carriers. Additionally, there was a close association between premature atherosclerotic

cardiovascular events and CHIP with a quadruple risk increase in patients younger than 50 years.[31] Interestingly, certain groups have suggested that a low degree of chronic inflammation in patients with risk factors for atherosclerosis may promote somatic changes in hematopoietic cell lines and eventually CHIP.[32, 33] Among a large number of mutation-susceptible loci, Tet methylcytosine dioxygenase 2 (TET2) appears to be the most commonly affected gene leading to CHIP. Indeed, TET2 mutations have been shown to promote clonal hematopoietic expansion and accelerate atherosclerosis in hyperlipidemic mice.[34] In animal models, TET2 deficient macrophages induced the expression of the NLRP3 inflammasome leading to overproduction of IL-1b (thereby favoring atherogenesis).[35]

Several other disease processes may potentially propagate a sustained cellular inflammatory response that promotes plaque formation. For instance, dyslipidemia and reduced cholesterol efflux [36] as well as hyperglycemia [37] have been associated with monocyte production at the hematopoietic level. Additionally, oxidized LDL promotes the release of epigenetically modified monocytes capable of sustaining prolonged inflammatory response. Monocytes and macrophages with these phenotypic changes appear to react with sustained responses to noxious stimuli, hence demonstrating an adaptive (i.e long lasting, memory-driven) rather than an innate immune response behavior (i.e., a first line defense). Since monocytes have a very limited life span (hours to days), the adaptive response to stimuli (named trained immunity) suggests that epigenetic changes may be occurring at the hematopoietic precursor cell level.[38]

CHAPTER 3

Histopathology of atheromatosis

3.1 Seminal events in the development of atherosclerotic plaques

3.1.1 Calcification

With the progress of time, several atherosclerotic plaques tend to regionally calcify. The process of atherosclerotic calcification occurs due to dysregulation and impaired clearance of calcium deposition. Interestingly, much of the mineralization process in atherosclerotic plaques closely resembles physiologic ossification.[39] Microscopic or spotty calcification promotes plaque instability and may predispose to rupture and thrombosis. On the other hand, bulky calcifications may reduce the risk of plaque rupture.[40]

3.1.2 Plaque rupture

Vulnerable plaques tend to have extensive lipid cores which are covered by a thin fibrous cap (<60 μm) and are therefore susceptible to rupture.[41] On the other hand, *stable plaques* are characterized by limited lipid accumulation and have thick fibrous caps. Defects in the extracellular matrix that overlies the lipid core of the atherosclerotic plaque can lead to the

formation of an overlying fibrous cap, and a fissure can form in this structure. Inflammatory cascades can impede synthesis of interstitial collagen by smooth muscle cells in the atherosclerotic plaque, impairing the ability of these cells to maintain the skeleton of the fibrous cap.[42] Activated inflammatory cells can also recruit matrix metalloproteinases (MMPs) and other interstitial collagenases specialized in degrading the key structural components of the fibrous cap of the lesion.[43]

Rupture of an atherosclerotic plaque exposes the contents of the interior of the plaque to blood. Thrombogenic material in the plaque core, especially tissue factor produced by macrophages and smooth muscle cells, can subsequently activate coagulation cascades leading to acute thrombosis. Depending on the insulted end-organ, thrombotic events could result in acute coronary syndromes, CVAs, acute peripheral arterial occlusion, and acute mesenteric ischemia to name a few.

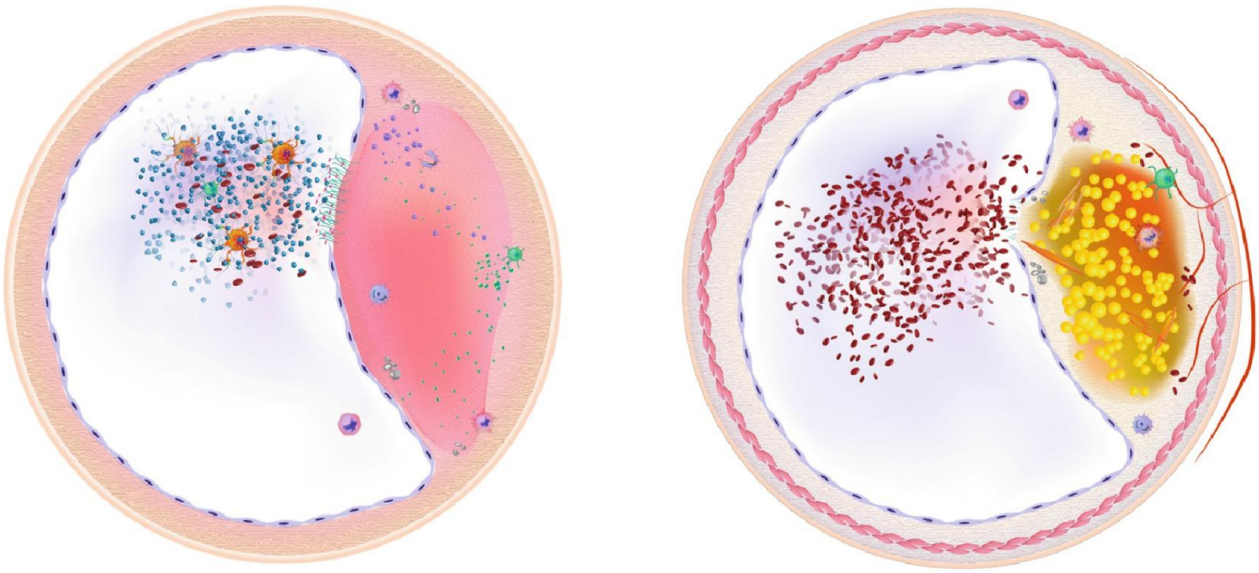
A growing body literature has implicated neutrophil extracellular traps (NETs) in vascular clotting.[44] NETs consist of strands of deoxyribonucleic acid (DNA) that bind leukocyte granular enzymes and proteins such as tissue factor. When neutrophils interact with these complexes, they undergo a specialized form of cell death known as NETosis. Ultimately, blood clots contain fibrin strands, clumps of activated platelets, and NETs that can propagate thrombus formation and amplify intimal injury.[45]

3.1.3 Plaque erosion

Effective management of atherosclerosis, including lipid-lowering interventions, anti-hypertensives, tight glycemic control, and smoking cessation, enable plaque stabilization.

Nevertheless, the incidence of acute thrombotic events has not declined. Although, plaque ruptures still occur, plaque erosion is emerging as a significant culprit of acute thrombosis. The pathophysiology of plaque erosion remains poorly understood. Emerging evidence suggests that innate immune reactions involving engagement of pathogen-associated molecular patterns (PAMPs) receptors, such as TLR 2, as well as the participation of neutrophils as amplifiers of the local thrombotic process may contribute to this type of plaque-related complication.[46] Lastly, NETs may propagate thrombosis during acute coronary syndromes, particularly those caused by intimal erosion.[46]

Many mural thrombi may not cause complete vessel occlusion or may undergo lysis by endogenous fibrinolytic defenses. The resolving thrombus constitutes a source of transforming growth factor- β (TGFβ) and platelet- derived growth factor derived by activated platelets. This environment can stimulate smooth muscle cell migration and extracellular matrix production. In turn, these processes lead to increased lesion volume and eventual encroachment upon the arterial lumen. Histopathological studies of advanced human atherosclerotic plaques showed “buried caps” that provide evidence of prior rupture and healing. Plaques that lack a well-defined lipid core and have abundant rather than sparse extracellular matrix can provoke coronary thrombi due to superficial erosion. Atheromata complicated by erosion also tend to have a thin, friable fibrous cap and minimal intralesional inflammatory components. The clots associated with superficial erosion have characteristics of platelet- rich “white” thrombi (**Figure G3.1**). By contrast, “red” thrombi are rich in fibrin and are abundant in trapped erythrocytes (distinctive in plaque rupture).[47] From a clinical standpoint, plaque ruptures more commonly lead to non-STEMI myocardial infarctions compared to full transmural ischemic cardiac events.



Feature	Erosion	Rupture
Fibrous cap	Intact	Ruptured
Morphology	Fibrotic ± deep lipid	Lipid-rich ± necrotic core
Composition	Proteoglycans, hyaluronans	Collagen poor
Inflammation	Minimal (secondary neutrophil activation)	High (macrophages)
Smooth muscle	Abundant	Absent or apoptotic
Thrombus	Platelet-rich	Erythrocyte and fibrin-rich
Clinical presentation	NSTE-ACS > STEMI	STEMI > NSTE-ACS

Figure G3.1. Plaque rupture vs erosion *(adapted from Thondapu V. et al. Cardiovascular OCT Imaging. p.79-89)*

3.2 Histologic classification of atherosclerotic plaques

In the late 1990s, the Council on Arteriosclerosis of the American College of Cardiology issued their landmark histological classification of atherosclerotic lesions.[48] Type I (initial) lesions contain enough atherogenic lipoprotein to elicit an increase in macrophages and form foam cells. As seen in more advanced lesion types, the changes are more marked in arterial sites with adaptive intimal thickening. These are present at fixed locations from birth, do not obstruct the

lumen and represent adaptations to local mechanical forces. Type II lesions consist primarily of layers of macrophage foam cells and lipid-laden smooth muscle cells and include lesions grossly designated as fatty streaks. In addition to the lipid-laden cells of type II, type III (or intermediate) lesions contain scattered collections of extracellular lipid droplets and particles that disrupt the coherence of intimal smooth muscle cells. This extracellular lipid deposit is the immediate precursor of the larger, confluent, and more disruptive core of extracellular lipid that characterizes type IV lesions (atheromas; **Figure G3.2**). Beginning around the fourth decade of life, lesions that usually have a lipid core may also contain thick layers of fibrous connective tissue (type V lesion/fibroatheromas) – some of which are heavily calcified (type Vb), while others consist mainly of fibrous connective tissue and have little or no accumulated lipid/calcium (type Vc). Lastly, atheromas containing fissures, hematomas, and thrombi are classified as type VI or complicated lesions.

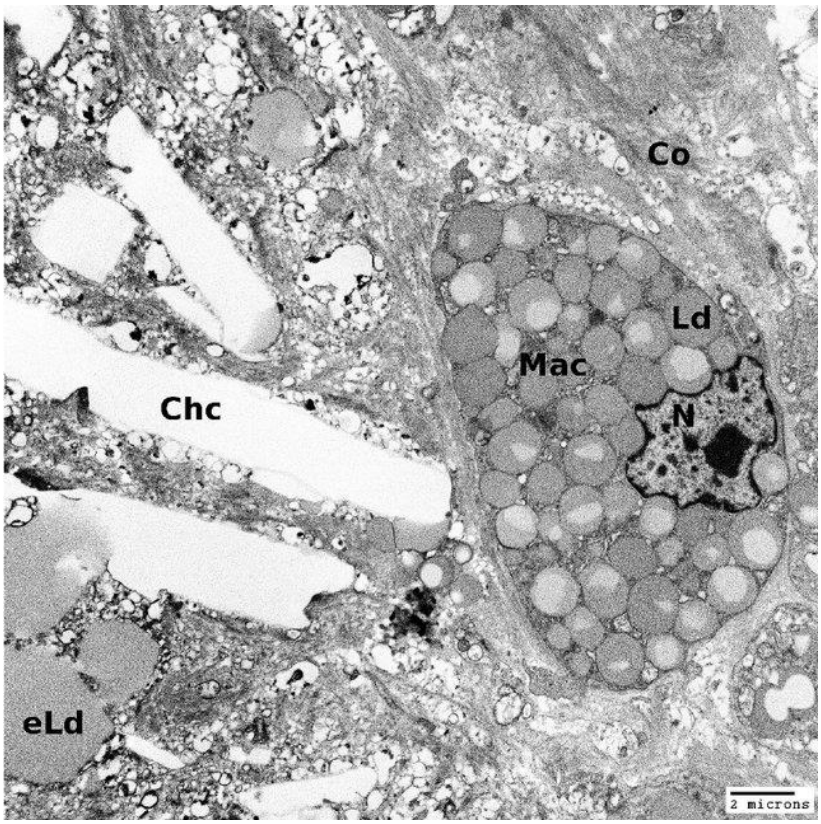


Figure G3.2.

Electron micrograph of an atherosclerotic plaque.

Mac: lipid-laden macrophage (foam cell); N, nucleus; Ld, intracellular lipid droplet; eLd, extracellular lipid droplet; Chc, cholesterol crystal; Co, collagen fiber *(adapted from Beaufrere H. et al. JAMS. Dec 2011. 25(4):266-76)*

CHAPTER 4

Inflammatory biomarkers in atheromatosis

4.1 C-reactive protein (CRP)

CRP is an annular pentameric protein found in blood plasma. Its circulating concentrations rise in response to inflammation. CRP is an acute-phase reactant of hepatic origin that increases following IL-6 secretion by macrophages and T cells. It binds to lysophosphatidylcholine on the surface of dead or dying cells in order to activate the complement system via C1q. CRP also enhances opsonin-mediated phagocytosis by macrophages (which express a receptor for it). The *CRP* gene is located on chromosome 1 (1q23.2). It is a member of the small pentraxins family. The monomer has 224 amino acids, and molecular mass of 25,106 Daltons. The complete protein is composed of five monomers and has a total mass of approximately 120,000 Daltons. In the serum, soluble CRP assembles into a stable pentameric structure with a discoid shape.[49]

CRP constitutes the most extensively studied biomarker in relation to the development of inflammation, coronary artery disease, and cardiovascular prognosis. Nevertheless, CRP production in response to vascular inflammation is quite small. Therefore, high-sensitivity CRP (hs-CRP) assay methods have been used to correlate patient outcomes with small changes in CRP concentrations. In a large meta-analysis of 160,309 subjects each standard deviation increase in log-normalized hs-CRP was associated with a multivariate adjusted relative increase in risk of 1.37

for future coronary artery disease (95% confidence interval [CI] 1.27-1.48) and 1.55 (95% CI interval 1.37-1.76) for future cardiovascular mortality. Adjusted for traditional risk factors, high sensitivity CRP levels below 1, between 1-3, and over 3 mg/L were associated with decreased, moderate, and increased relative risk, respectively. [50]

In the Justification for the Use of Statins in Prevention: an Intervention Trial Evaluating Rosuvastatin (JUPITER) trial, the investigators showed that rosuvastatin reduced the rate of first myocardial infarction, stroke, or confirmed cardiovascular death by 47% in patients with low-density lipoprotein-C levels of <130 mg/dl and hsCRP of >2 mg/L (hazard ratio: 0.53; 95% confidence interval: 0.40 to 0.69; $p < 0.00001$).[51] However, given that no patients with a baseline hs-CRP < 2 mg/L were enrolled, there is no data to suggest that rosuvastatin may not have been equally effective even in patients with even lower CRP levels. Although, elevated hs-CRP has been shown to be predictive of plaque rupture and vascular thrombosis, its impact on predicting future cardiovascular events in patients who are on optimal preventive medications (including anti-platelet agents and statins) is weak in secondary prevention studies.[52]

4.2 Interleukin 6 (IL-6)

IL-6 is secreted by macrophages in response to PAMPs. These bind to an important group of detection molecules of the innate immune system, called pattern recognition receptors (PRRs) and include TLRs. PRRs are present on the cell surface and intracellular compartments of macrophages and dendritic cells. They induce intracellular signaling cascades that incite inflammatory cytokine production. IL-6 signals via a cell-surface type I cytokine receptor complex consisting of the ligand-binding IL-6Rα chain (CD126), and the signal-transducing component

gp130 (also called CD130). CD130 is the common signal transducer for several cytokines including leukemia inhibitory factor (LIF), ciliary neurotropic factor, oncostatin M, IL-11 and cardiotrophin-1, and is almost ubiquitously expressed in most tissues. In contrast, the expression of CD126 is restricted to certain tissues. As IL-6 interacts with its receptor, it triggers the gp130 and IL-6R proteins to form a complex, thus activating the receptor. These complexes bring together the intracellular regions of gp130 to initiate a signal transduction cascade through seminal transcription factors, such as Janus kinases (JAKs) and Signal Transducers and Activators of Transcription (STATs). IL6 exerts its anti-inflammatory action by inhibiting TNF-alpha and IL-1 as well as by activating IL-1ra and IL-10.

Notably, IL-6 signaling has been shown to influence plaque destabilization and microvascular dysfunction. It can also predict adverse outcomes in the setting of acute ischemia. Indeed, IL-6 plasma levels equal or greater than 5 ng/L have been associated with 300% increased mortality risk in patients with unstable angina.[53] Furthermore, IL-6 levels have been shown to correlate with the severity of acute coronary syndromes according to both SYNTAX score I ($r = 0.479$, $P < 0.001$) and II ($r = 0.305$, $P < 0.001$). On the same note, IL-6 levels seem to be independently predictive of intermediate-high SYNTAX I scores (AUC: 0.806) and high SYNTAX II values (0.624).[54]

4.3 Additional inflammatory biomarkers in atheromatosis

Myeloperoxidase (MPO) is a peroxidase enzyme that is abundantly expressed in neutrophils and plays a seminal role in their microbicidal action. MPO causes oxidative modification of LDL cholesterol thus promoting atherogenesis. Initial plasma MPO levels at the

time of admission have been shown to predict the risk of myocardial infarction, even in patients who are negative for troponin T (<0.1 ng per milliliter) at baseline ($p<0.001$). MPO levels at presentation may also be able to predict the risk of major adverse cardiac events (myocardial infarction, the need for revascularization, or death) within 30 days after presentation ($P<0.001$).[55] In a multivariate analysis of patients with acute coronary syndrome (ACS), MPO levels (HR 2.11; $P=0.008$) were shown to be independent predictors of six-month cardiovascular outcomes irrespective of troponin, CRP, and other inflammatory markers such as vascular endothelial growth factor and soluble CD40 ligand.[56]

Lipoprotein-associated phospholipase A2 (Lp-PLA2) is a calcium independent phospholipase A2 enzyme secreted by leukocytes. It is associated with circulating LDL and macrophages in atherosclerotic plaques. Lp-PLA2 generates two proinflammatory mediators, lysophosphatidylcholine and oxidized non-esterified fatty acids. These play a major role in the formation of atherosclerotic lesions. There is growing evidence from primary and secondary prevention studies that this inflammatory marker may be an independent predictor of cardiovascular events. Evidence also points towards its role as a potential marker of endothelial dysfunction.[57]

A myriad of additional inflammatory biomarkers may be potentially useful in predicting the severity of atheromatosis, including neopterin, serum amyloid A, pregnancy-associated plasma protein-A, monocyte chemoattractant protein 1, growth differentiation factor-15, galectin-3 binding protein, fibrinogen, pentraxin 3 and soluble ST2. Nevertheless, no compelling evidence exists to date that could justify their integration into clinical diagnostic protocols.[58]

CHAPTER 5

Anti-inflammatory therapy in atheromatosis

In recent years, a variety of immunomodulators have been assessed as potential adjuncts to the standard treatment of atheromatosis. Furthermore, several of the key medications used in cardiovascular disease seem to possess off-target anti-inflammatory properties.[59]

5.1 Current off-target anti-inflammatory therapies

5.1.1 Statins

Statins afford cardiovascular protection by reducing LDL levels through the inhibition of HMG-CoA (3-hydroxy-3 -methylglutaryl-Coenzyme A) reductase. That said, there is ample data suggesting that statins also decrease cardiovascular inflammation. First, statins inhibit prenylated protein production and the mevalonate pathway while inducing Krüppel-like factor 2 (KLF 2) and NOS (nitric oxide synthase) expression.[60] Statins also reduce endothelial cell activation and inhibit the induction of major histocompatibility complex Class II expression by interferon (IFN)-g thereby decreasing T-cell activation.[61]

5.1.2 Aspirin and anti-platelet therapy

Aspirin and other antiplatelet drugs inhibit P-selectin which in turn reduces the release of inflammatory chemokines. Low-dose aspirin also triggers the synthesis of 15-epi-lipoxin A4, which mediates NOS synthesis and limits endothelial cell activation and leukocyte recruitment.[62] In human aortic endothelial cell lines, intracytosolic NLRP-1 expression is also attenuated by aspirin, without direct platelet-endothelial cell interaction.[63] A landmark study by Brigham and Women's Hospital, showed a 55% reduction in the risk for myocardial infarction in healthy men with high CRP levels taking aspirin.[64]

Moreover, P2Y12 inhibitors reduce platelet release of pro-inflammatory α-granule contents and obstruct the formation of pro-inflammatory platelet-leukocyte aggregates. Clinical evidence shows that P2Y12 inhibition by clopidogrel is associated with a reduction in platelet-related mediators of inflammation, such as soluble P-selectin and CD40L. Compared to aspirin alone, the addition of clopidogrel, also significantly reduces markers of systemic inflammation such as TNF-a and CRP following ACS. The more potent thienopyridine P2Y12 inhibitor, prasugrel, has been shown to decrease platelet P-selectin expression and platelet-leukocyte aggregate formation more robustly compared to clopidogrel.[65]

The Study of Platelet Inhibition and Patient Outcomes (PLATO) suggested that the novel P2Y12 inhibitor ticagrelor might predispose less to pulmonary infections and sepsis compared to clopidogrel in patients with ACS.[66] Ticagrelor is a more potent P2Y12 inhibitor than clopidogrel and also inhibits cellular adenosine uptake via equilibrative nucleoside transporter (ENT) 1,

whereas clopidogrel does not. For all of the aforementioned reasons, aspirin and P2Y12 inhibitors seem to be extremely useful tools in reducing cardiovascular inflammation.

5.2 Canakinumab

The randomized, double-blind Canakinumab Anti-Inflammatory Thrombosis Outcomes (CANTOS) trial, investigated canakinumab, a monoclonal antibody targeting interleukin-1β.[67] The study involved 10,061 patients with prior myocardial infarction and a high-sensitivity C-reactive protein level of 2 mg/lt or more. The patients were randomized to receive one of 3 canakinumab doses (50 mg, 150 mg, and 300 mg) administered subcutaneously every 3 months or placebo. All patients received standard of care therapy and serum LDL levels at the time of enrollment had to be within guideline-dictated limits.

The primary efficacy endpoint of major adverse cardiovascular events (MACEs): non-fatal myocardial infarction, non-fatal stroke, or CVD was achieved in the 150mg study arm (hazard ratio [HR] 0.85; 95% CI, 0.74 to 0.98; $p<0.05$) dose. The secondary endpoint included a combination of CVD, non-fatal myocardial infarction and stroke plus hospitalization for unstable angina leading to urgent revascularization and was again significantly lower with the 150 mg regimen (HR 0.83; 95% CI, 0.73 to 0.95; $p = 0.005$), (**Figures G5.1A-B)**. It should be emphasized that canakinumab was associated with a higher incidence of fatal infections than placebo (0.31 events x 100 person years for all combined doses vs. 0.18 events x100 person years for placebo; $p = 0.02$). That said, canakinumab dosing did not influence all-cause or cardiovascular mortality.

The CANTOS trial group also performed prespecified subanalyses to identify which patient groups may benefit the most from canakinumab and whether reductions in hsCRP levels

correlate with clinical benefits.[68] Compared to placebo, MACEs were significantly reduced in patients with an hs-CRP level <2 mg/L after 3 months of treatment (HR: 0.75 (95%CI 0.66-0.85; p<0.0001) but not in those with an hsCRP >2 mg/L. Among patients with hs-CRP<2 mg/L there was also a 31% reduction in cardiovascular and all-cause mortality (p <0.0004 and p<0.0001, respectively).

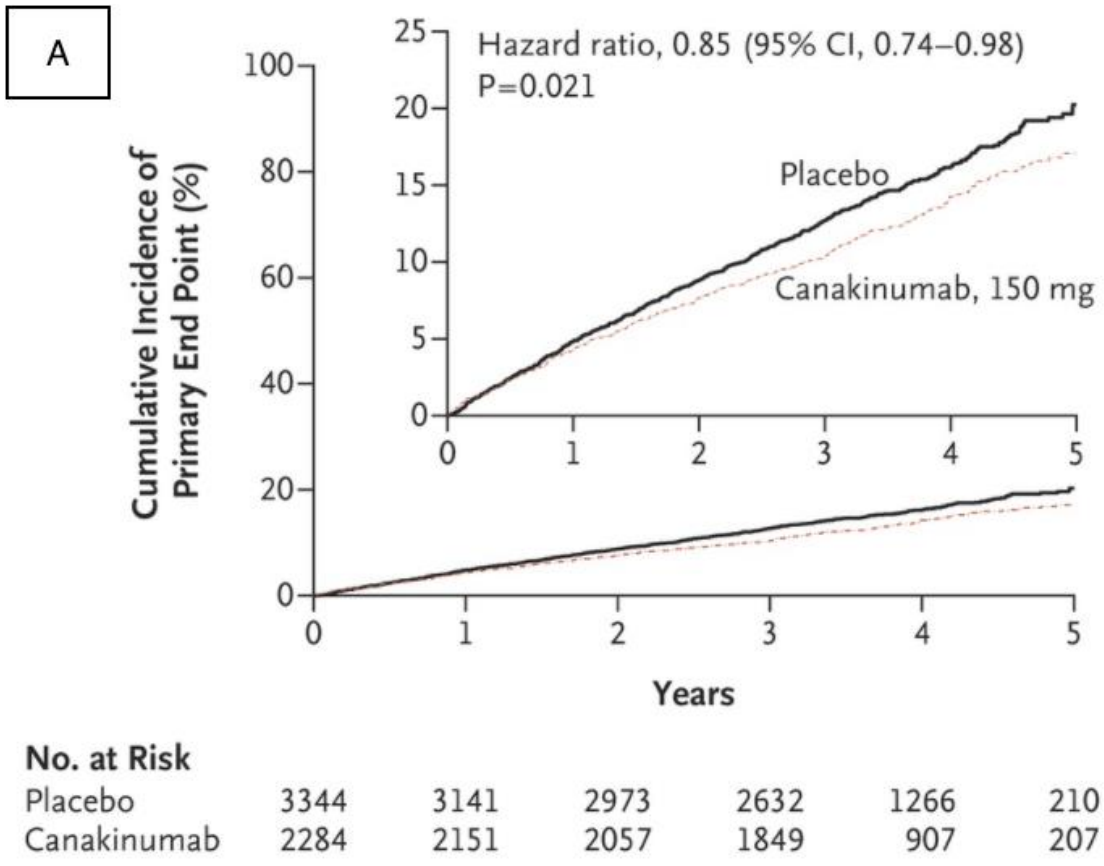


Figure G5.1A. In the CANTOS trial, the group receiving the 150-mg dose of canakinumab met the threshold for statistical significance for the primary cardiovascular end point *(adapted from*

Ridker P.M., et al., Anti-inflammatory Therapy with Canakinumab for Atherosclerotic Disease. N Engl J Med, 2017. 377(12): p. 1119-1131.)

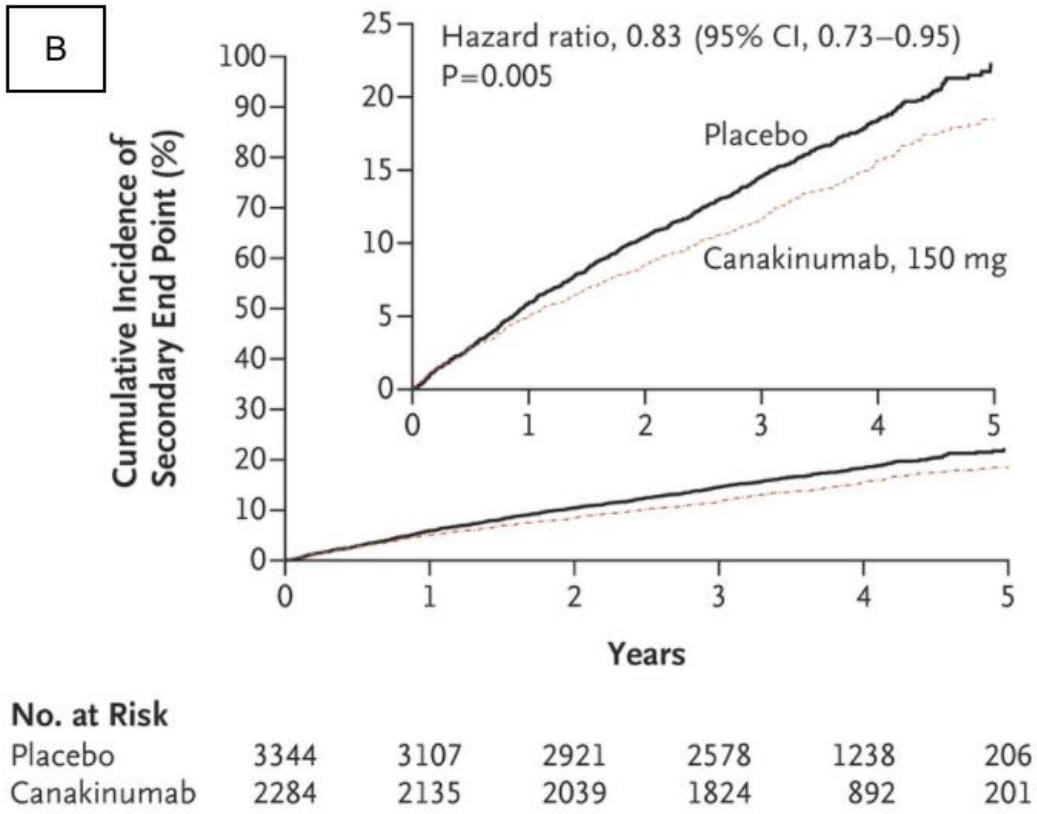


Figure G5.1B. In the CANTOS trial, the group receiving the 150-mg dose of canakinumab also required significantly less hospitalizations for unstable angina leading to urgent revascularization *(adapted from Ridker P.M., et al., Anti-inflammatory Therapy with Canakinumab for Atherosclerotic Disease. N Engl J Med, 2017. 377(12): p. 1119-1131.)*

A subsequent analysis of CANTOS data compared nearly 2,000 patients with chronic kidney disease (CKD: estimated glomerular filtration rate <60 ml/min/1.73m2) with the remaining approximately 8,000 patients enrolled in CANTOS.[69] Canakinumab reduced MACEs in CKD patients and was particularly effective in those who achieved a level of hs-CRP<2 mg/L after the first drug dose. In patients with hs-CRP <2 mg/L, cardiovascular and all-cause mortality were also significantly reduced (with no adverse laboratory or clinical renal events).

5.3 Colchicine

Colchicine is an inexpensive, readily available, *per os* medication, with strong anti-inflammatory effects. It was originally obtained as an extract from Colchicum autumnale. Colchicine inhibits tubulin polymerization and microtubule generation and modulates several cellular adhesion molecules such as selectins, leukocyte function-associated antigen-1, and the inflammasome. [70, 71] Classical use indications for colchicine include acute gout attacks, familial Mediterranean fever, and acute pericarditis.

In the randomized-controlled LoDoCo (low dose colchicine) trial, 0.5mg/day colchicine was tested in over 500 patients with stable CAD.[72] The composite primary outcome of acute coronary syndrome, out-of-hospital cardiac arrest, or non-cardio-embolic ischemic stroke occurred in 5.3% of patients on colchicine versus 16% of patients on the placebo arm (HR: 0.33; 95% CI, 0.18 to 0.59; $p < 0.001$, **Figure G5.2**). Therefore, the LoDoCo trial provided compelling evidence suggesting that colchicine may be beneficial in preventing recurrent cardiovascular events in patients with stable angina.

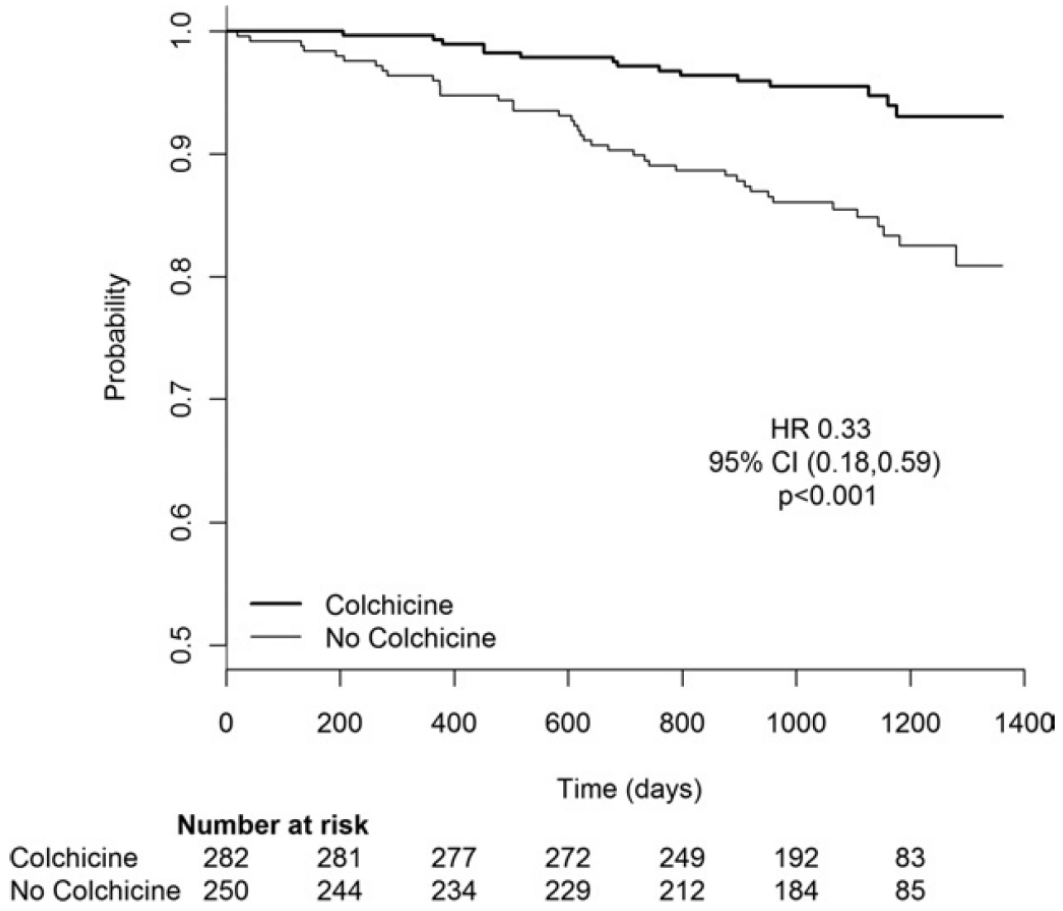


Figure G5.2. In the LoDoCo trial, freedom from the composite primary cardiovascular outcome was significantly higher in patients with stable CAD receiving low dose colchicine *(adapted from Nidorf S.M., et al. Low-dose colchicine for secondary prevention of cardiovascular disease. J Am Coll Cardiol, 2013. 61(4): p. 404-410.)*

In light of these encouraging preliminary results, the Colchicine Cardiovascular Outcomes Trial (COLCOT) was designed to examine the effects of low dose colchicine on cardiovascular events in over 4,500 patients with an acute coronary syndrome.[73] In the COLCOT trial, patients

were randomized within 30 days of an acute coronary syndrome and after planned percutaneous revascularization to the standard of care and colchicine 0.5 mg/day or placebo.

The primary efficacy end point was a composite of death from cardiovascular causes, resuscitated cardiac arrest, myocardial infarction, stroke, or urgent hospitalization for angina leading to coronary revascularization (**Figure G5.3**). Patients were followed for a median of 22.6 months. The primary end point occurred in significantly less patients in the colchicine (5.5%) versus the placebo group (7.1%). (HR: 0.77; 95% CI: 0.61 to 0.96; p = 0.02).

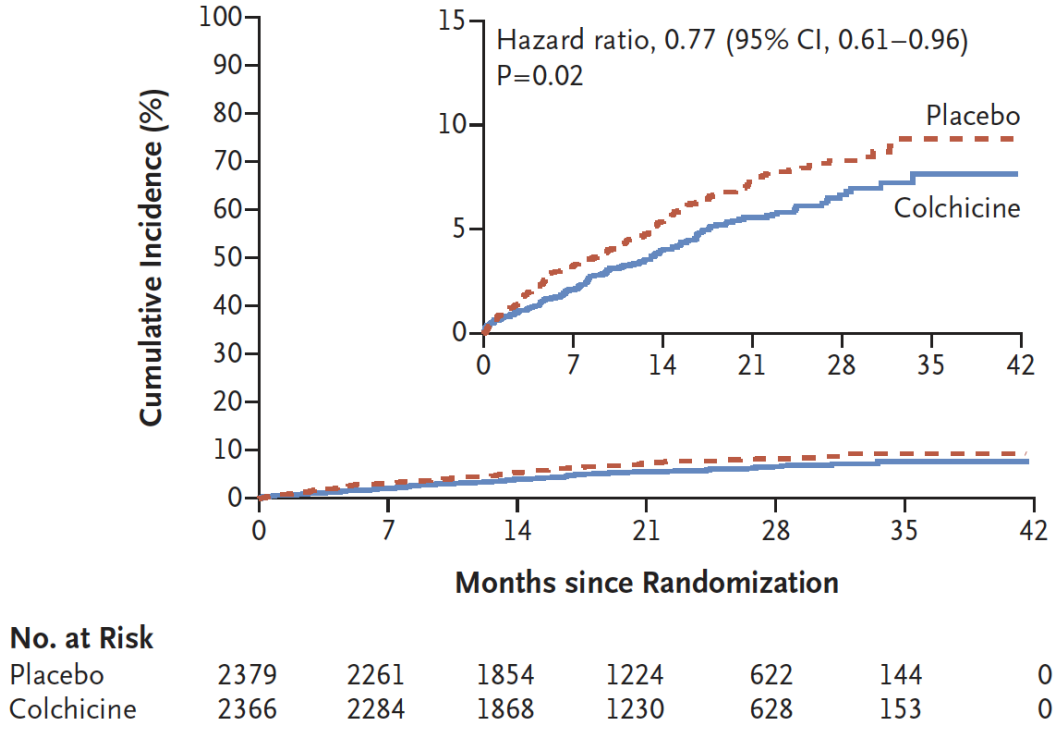


Figure G5.3. In the COLCOT trial, low dose colchicine in the setting of an acute coronary event led to a statistically significant reduction in death from cardiovascular causes, resuscitated cardiac arrest, myocardial infarction, stroke, or urgent hospitalization for angina leading to coronary revascularization *(adapted from Tardif J.C., et al. Efficacy and Safety of Low-Dose Colchicine after Myocardial Infarction. N Engl J Med, 2019. 381(26): p. 2497-2505.)*

The HR were 0.84 (95% CI, 0.46 to 1.52) for death from cardiovascular causes, 0.83 (95% CI, 0.25 to 2.73) for resuscitated cardiac arrest, 0.91 (95% CI, 0.68 to 1.21) for myocardial infarction, 0.26 (95% CI, 0.10 to 0.70) for stroke, and 0.50 (95% CI, 0.31 to 0.81) for urgent hospitalization for angina leading to coronary revascularization. Although reasonable to wonder, the therapeutic effect of colchicine on pericarditis did not seem to confound COLCOT outcomes for two reasons. First, postinfarction pericarditis usually occurs within the first few days after a myocardial infarction. Second, the average time from the index myocardial infarction to randomization was 13.5 days.

Importantly, the cardiovascular benefits of colchicine were at the very least non-inferior to those seen with canakinumab in the CANTOS trial [67]. Additionally, the risk of septic shock was not increased in the COLCOT trial, thereby making an argument for colchicine as a preferable agent compared to canakinumab.

5.4 NLRP3 inhibitors

MCC950 and other small-molecule inhibitors of NLRP3 (NLR family pyrin domain containing 3), have been developed to treat a variety of inflammasome-driven diseases including CAD. MCC950 was recently shown to reverse the accelerated atherosclerosis phenotype in mice with myeloid TET2 deficiency [34] and may be particularly beneficial in CHIP-associated atherosclerotic disease. A trial to assess MCC950 and other selective small-molecule compounds in patients with ischemic heart disease is under way by the time of this writing. NLRP3 inhibition would also lead to a reduction of active IL-18 and IL-1b. These changes could potentially provide

extra protection against atherosclerotic disease.[74] The most important side-effect of NLRP3 inhibitors is the increased risk of infections.

5.5 Fibrates

Pharmacologically speaking, fibrates constitute amphipathic carboxylic acids which are used as adjuncts to dietary modifications in primary hypercholesterolemia, mixed dyslipidemia, and severe hypertriglyceridemia. Fibrates stimulate peroxisome proliferator activated receptor (PPAR) alpha, which regulates the metabolism of triglycerides (TG) and high-density lipoprotein (HDL). As a result, synthesis of fatty acids, TG and VLDL is reduced. On the other hand, lipoprotein lipase, which catabolizes TG, is upregulated. In addition, production of Apo A1 and ATP binding cassette A1 is enhanced, leading to increased reverse cholesterol transport via HDL.

Although the impact of fibrates on LDL varies, fibrates can reduce TG by up to 50% and increase HDL-C by up to 20%. Fibrates have been shown to decrease the incidence of cardiovascular events by approximately 10%, albeit their benefit on cardiovascular and all-cause mortality remains debatable.[75] Common side effects include gastrointestinal disturbance, rhabdomyolysis, acute kidney injury, cholestasis and gallstones.

An increasing body of literature has provided compelling evidence that fibrates bear anti-inflammatory properties.[76, 77] Indeed, PPARα blocks NF-kB thereby halting the expression of a cascade of inflammatory genes.[78] Fenofibrate, in particular, has also been shown to inhibit the production of IL-6, IL-1β, and cyclooxygenase 2 (COX-2) -which is expressed by endothelial cells, smooth muscle cells, and macrophages in human atherosclerotic lesions.[76, 79]

5.6 N-acetylcysteine (NAC)

NAC is the N-acetyl derivative of the amino acid L-cysteine and serves as a precursor in the formation of the antioxidant glutathione. Clinical indications of NAC include paracetamol overdose and mucus-producing bronchopulmonary disorders, either acute (e.g., pneumonia) or chronic (e.g chronic obstructive pulmonary disease). Although, injectable NAC may be more potent, it can lead to significant side effects such as bronchospasm, hypotension, severe nausea, extensive mucocutaneous reactions, and anaphylaxis. Therefore, oral preparations of NAC are strongly recommended whenever its use is deemed clinically useful.

From a biochemistry perspective, the thiol (sulfhydryl) group confers antioxidant effects and is able to scavenge ROS and other free radicals.[80] NAC inhibits TNF-α activation of NF-κB by inducing structural changes in the TNF-α receptor, thus decreasing the receptor's affinity for this mediator. Oral NAC supplementation has also been shown to decrease CRP and IL-6 plasma levels in a non-linear fashion.[81]

Experimental data from animal models have shown that NAC may shield aortic tissue against atherogenesis. NAC treatment in the drinking water of diabetic Apolipoprotein-E knockdown (ApoE-/-) mice for 12 weeks decreased the size of aortic atherosclerotic lesions (**Figure G5.4**). This was associated with a reduction in methylglyoxal-dicarbonyl stress and oxidative stress, as indicated by decreased serum malondialdehyde levels, as well as by increased superoxide dismutase-1 and glutathione peroxidase-1 levels in the diabetic aorta. Endothelial damage was also corrected by NAC, as indicated by an increase in the expression levels of phosphorylated(p-) Akt and p-endothelial nitric oxide synthase in the aorta, as well as by nitric oxide in the serum.[82]

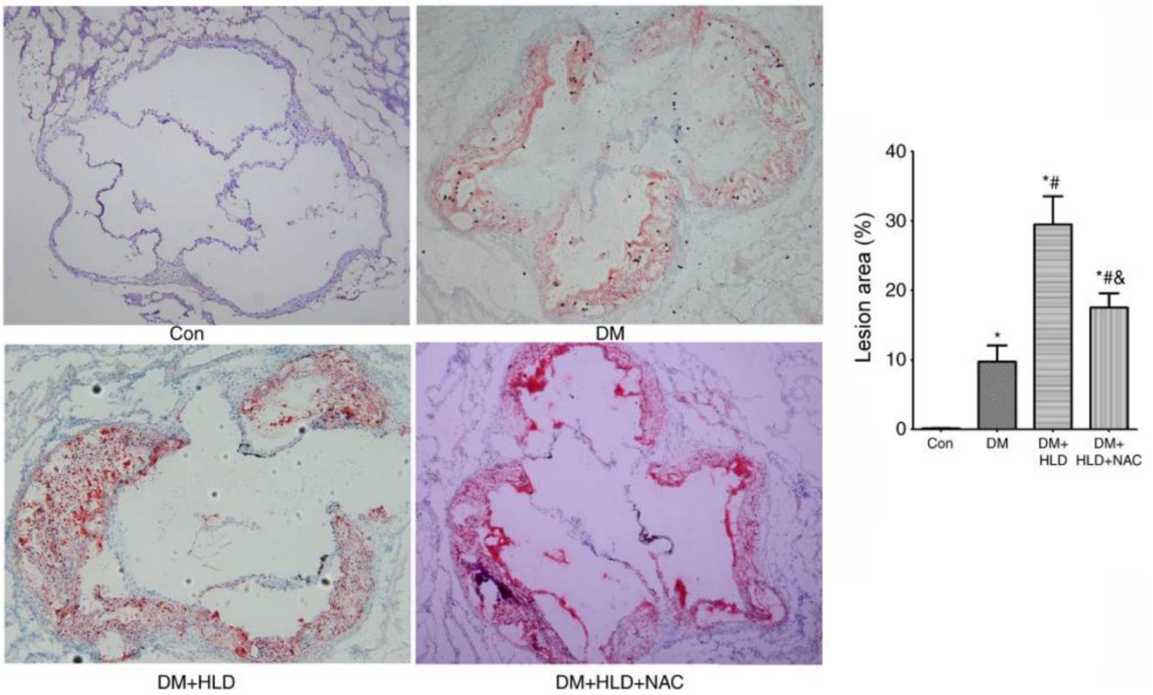


Figure G5.4. Atherosclerotic lesion areas in the aortic root were stained with oil red O (magnification, ×100) in *ApoE*$^{-/-}$ mice (Con group), STZ-injected *ApoE*$^{-/-}$ mice (DM group), STZ-injected *ApoE*$^{-/-}$ mice fed a HLD (DM + HLD group), and STZ-injected *ApoE*$^{-/-}$ mice fed a HLD and administered NAC-containing water (DM + HLD + NAC group). *P<0.05 vs. Con group; $^{\#}$P<0.05 vs. DM group; $^{\&}$P<0.05 vs. DM + HLD group (n=5/group). *ApoE*, apolipoprotein E; Con, control; DM, diabetes mellitus; HLD, high-lipid diet; NAC, N-acetylcysteine; STZ, streptozotocin *(adapted from Fang X., et al Mol Med Rep, 2021. 23(3).)*

5.7 Immunomodulators with little to no benefit in atheromatosis

Cardiovascular risk is increased in patients with autoimmune and rheumatologic disorders. Methotrexate, in addition to curtailing the secretion of pro-inflammatory cytokines, also influences cholesterol transport.[83] In-vitro studies have found that methotrexate inhibits the degradation of the reverse cholesterol transport proteins 27-hydroxylase and of ATP-binding cassette transporter

A1 (ABCA1) via activation of the A2A adenosine receptor.[83] Immunomodulators also exert their anti-atherogenic effects via reducing the expression of vascular adhesion molecules such as VCAM-1 and ICAM-1, which enhances overall endothelial function.[84]

To test methotrexate in CAD, the phase 3 Cardiovascular Inflammation Reduction Trial (CIRT), recruited 4,786 patients with previous MI or multivessel CAD who had either type 2 diabetes or metabolic syndrome. These patients were subsequently randomized to either low-dose methotrexate or placebo.[85] The trial was stopped after two years due to lack of difference between groups in the primary composite endpoint of MI, stroke, cardiovascular death, or unstable angina. A reason for the negative result is perhaps the inability of low dose methotrexate to reduce inflammation (no effect on hsCRP, IL-1b, or IL-6) in patients with CAD, particularly when median hsCRP levels at baseline (1.6 mg/l) are substantially lower in comparison with patients who present with flares of rheumatoid or psoriatic arthritis.

Multiple additional pathways have been explored as potential targets for the prevention and treatment of cardiovascular diseases (**Table G5.1**). No significant benefit has been proven from targeting oxidized LDL (succinobulol) [86], secretory phospholipase A2 (Veresplabdib) [87], lipoprotein-associated phospholipase A2 (Darapladib) [88], P-selectin (Inclacumab) [89], IL-1RI (Anakinra) [90], IL-1A (Xilonix) [91], or 5-lipoxygenase (Atreleuton) [92], p38 mitogen-activated protein kinase (Losmapimod).[93]

Table G5.1. Anti-inflammatory agents that failed to reduce cardiovascular risk significantly

Target	**Agent**	**Trial**	**Number of participants**

Oxidized LDL	Succinobulol	ARISE	6,144
sPLA2	Verespladib	VISTA-16	5,000
LpPLA2	Darapladib	STABILITY	15,000
LpPLA2	Darapladib	SOLID-TIMI 52	13,000
P-selectin	Inclacumab	SELECT-ACS	544
P-selectin	Inclacumab	SELECT-CABG	380
IL-1RI	Anakinra	IL-HEART	182
5-LO	Atreleuton	NCT00358826	191
p38 MAPK	Losmapimod	LATITUDE-TIMI	3,503
Dihydrofolate reductase inhibitor	Methotrexate	CIRT	4,786

sPLA2: secretory phospholipase A2; LpPLA2: lipoprotein-associated phospholipase A2; interleukin 1RI: IL-1RI; interleukin 1A: IL-1A; 5-LO: 5-lipoxygenase; MAPK: mitogen-activated protein kinase; ARISE: ; Aggressive Reduction of Inflammation Stops Events trial; VISTA-16: Vascular Inflammation Suppression to Treat Acute Coronary Syndrome; STABILITY: STabilization of Atherosclerotic plaque By Initiation of darapLadIb TherapY trial; SOLID-TIMI 52: Stabilization Of pLaques usIng Darapladib-Thrombolysis In Myocardial Infarction 52 trial; SELECT-ACS: P-Selectin Antagonist Inclacumab on Myocardial Damage After Percutaneous Coronary Intervention for Non-ST-Segment Elevation Myocardial Infarction trial; SELECT-CABG: Effects of P-Selectin Antagonist Inclacumab in Patients Undergoing Coronary Artery Bypass Graft Surgery trial; LATITUDE-TIMI: Losmapimod to Inhibit p38 MAP Kinase as a Therapeutic Target and Modify Outcomes After an Acute Coronary Syndrome trial; CIRT: Cardiovascular Inflammation Reduction Trial

CHAPTER 6

Shear stress regulates the expression of stem cell genes in atheromatosis

6.1 Omics studies of endothelial cell mechanoresponses

Several in vitro and in vivo studies have utilized microarrays and ribonucleic acid (RNA) sequencing to assess endothelial cell transcriptome under different flow conditions. [94, 95] Such approaches enable the discovery of unexpected new roles for molecules whose known functions do not make them obvious culprits for atherogenesis. Overall, these studies have shown that shear stress regulates multiple transcriptional pathways involved in inflammation, cell cycle, apoptosis, cell migration, protein oxidation, and the unfolded protein response. For example, messenger RNA (mRNA) profiling revealed upregulation of pro-inflammatory, antioxidant and stress-response molecules at atheroprone sites of the pig aorta.[96-98] Carotid partial ligation models in mice identified genes regulated by shear stress and discovered changes in genetic pathways involved in the regulation of cellular proliferation, architecture, and inflammation.[99] More recently, shear stress has been found to influence the expression of stem cell genes which are physiologically involved in embryonic development and angiogenesis.

Furthermore, methylome studies have also explored previously uncharted shear stress-regulated pathways. Indeed, carotid partial ligation mouse models [100] and in vitro studies using

pig endothelial cells [101] have revealed that blood flow epigenetically controls the expression of multiple genes by regulating genome-wide DNA methylation patterns. Differentially methylated regions have been associated with oxidative stress and the endoplasmic reticulum stress adaptive pathway, thereby implicating these processes in early atherogenesis.[101, 102]

6.2 Angiogenesis and vascular remodeling in atherogenesis

Alterations in signaling pathways due to disturbed flow in atheroprone regions closely mirror phenomena seen during fetal vascular development.[103] During embryogenesis, vasculature forms through two sequential, flow-sensitive processes: vasculogenesis and angiogenesis. Vessels initially form through vasculogenesis and subsequently undergo angiogenic sprouting to form a vascular plexus. These formations mature through pruning – a process involving apoptosis and migration of endothelial cells. The remodeled vasculature subsequently undergoes differentiation to form mature arteries and veins.[104]

Shear stress regulates multiple processes in vascular development including sprouting, endothelial cell apoptosis, endothelial cell migration and arterial fate specification.[105] This flow-dependent regulation ensures that angiogenic and vascular pruning processes are active in developing vascular beds as needed. On the other hand, these processes are deactivated in tissues that have mature vasculature.[106] In atheroprone regions, low shear stress forces cause inappropriate activation of developmental signaling pathways, leading to increased inflammation and vascular permeability, which are the hallmarks of early atherogenesis (**Figure G6.1**).[107, 108]

Most of these pro-angiogenic pathways are normally inactive in adult arteries exposed to high flow, but they can be activated at branches and curvatures that are exposed to low shear stress (**Figure G6.2**). Activation of dormant embryonic pathways is pathogenic because it promotes upregulation of cellular adhesion molecules, decreased NO production, disorganization of endothelial actin fibers, and shortening of the glycocalyx. These changes increase vascular permeability which ultimately leads to the accumulation of cholesterol and inflammatory cells in the arterial intima. The aforementioned conceptual framework explains how endothelial cells respond to low shear stress on a molecular level.

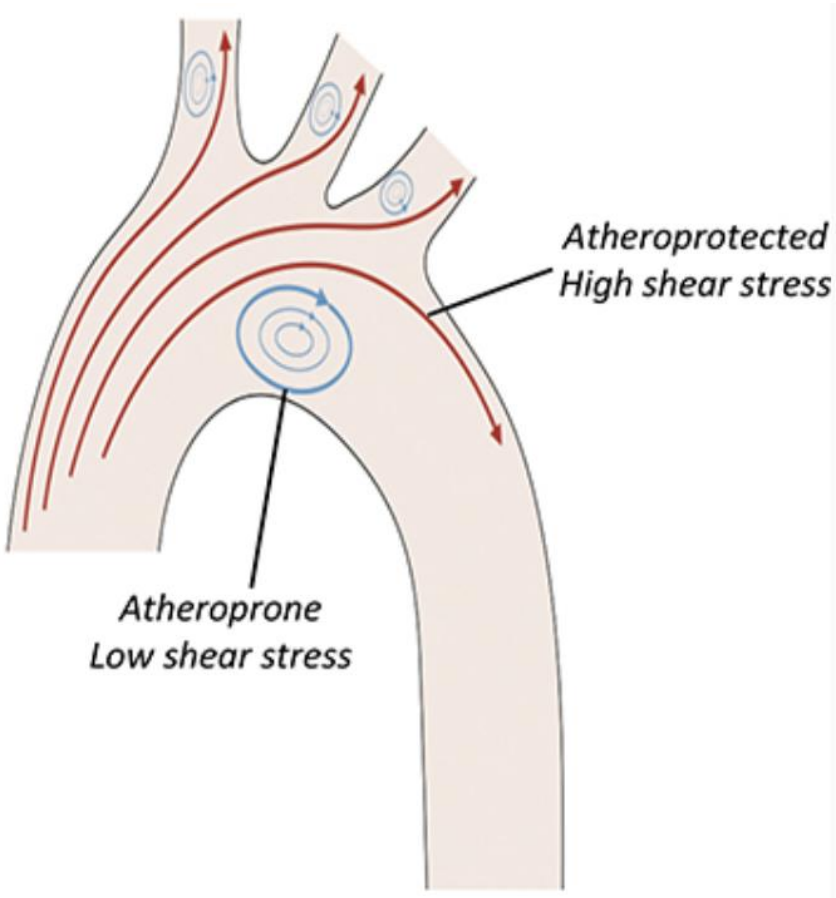


Figure G6.1 Low shear stress prevails in atheroprone regions *(adapted from Feng S., Bowden N., Fragiadaki M., Souilhol C., Hsiao S., Mahmoud M., et al., Mechanical Activation of Hypoxia-*

Inducible Factor 1a Drives Endothelial Dysfunction at Atheroprone Sites. Arterioscler Thromb Vasc Biol, 2017. 37(11): p. 2087-2101.)

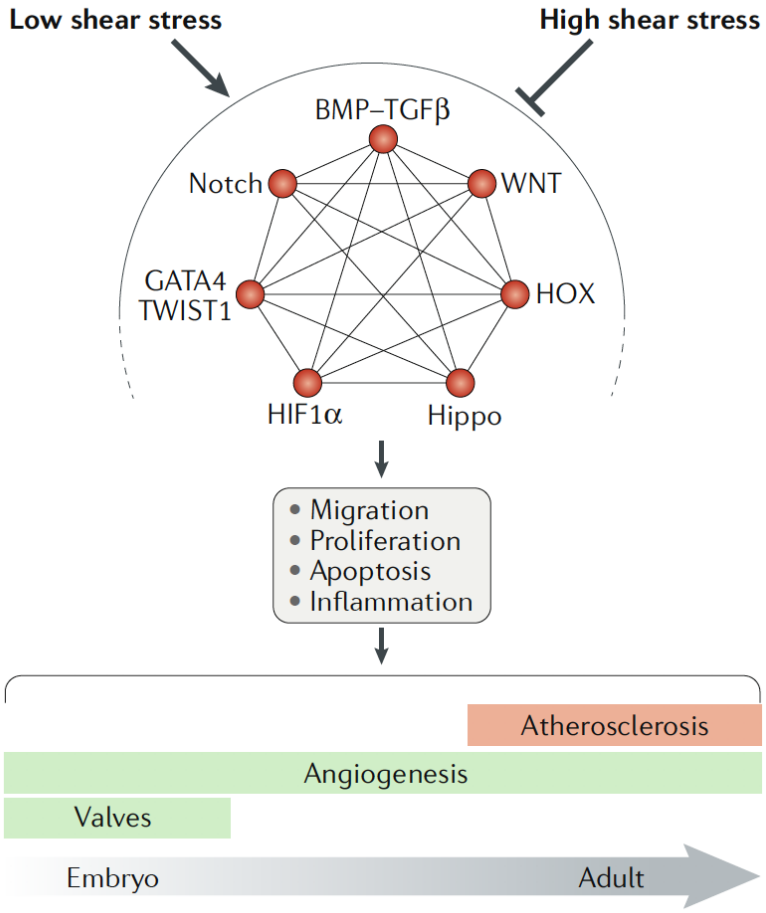


Figure G6.2. Shear stress regulates multiple signaling pathways with a classical role in embryonic development, including members of the bone morphogenetic protein (BMP)–transforming growth factor- β (TGFβ) superfamily, hypoxia- inducible factor 1α (HIF1α), Notch, Hippo–Yes-associated protein (YAP)–transcriptional co-activator with PDZ- binding motif (TAZ), WNT, transcription factor GATA4 and twist related protein 1 (TWIST1), *(adapted from Souilhol C., et al., Endothelial responses to shear stress in atherosclerosis: a novel role for developmental genes. Nat Rev Cardiol, 2020. 17(1): p. 52-63.)*

CHAPTER 7

Krüppel-like factor 4 (KLF4)

7.1 Structure and function of KLF4

Krüppel-like factor 4 (or gut-enriched KLF) is a member of the KLF family of zinc-finger transcription factors, which belongs to the SP1-like superfamily of transcription factors. The *KLF4* gene was originally isolated by Shields et al. in 1996 from a NIH3T3 cDNA library.[109] It is conserved among vertebrate species from zebrafish to humans. *KLF4* has been gaining increased traction since 2006 when it was found to be one of four Yamanaka factors (OCT-3/4 + SOX2 + KLF4 + c-MYC) required for the production of induced pluripotent stem cells (iPSCs).[110]

Structurally, KLF4 is characterized by three zinc finger motifs within its carboxyl terminal sequence. Within its amino terminus, KLF4 possess a transactivation domain (TAD) and adjacent to it, a repression domain, together which determine the specificity of KLF4's transcriptional regulating activity by interacting with other factors and modulating DNA binding efficiency. Two nuclear localization signals (NLS) have also been identified in KLF4. The first is directly adjacent to the most amino terminal-adjacent zinc finger motif and the second spans the first and half of the second zinc finger domains (**Figure G7.1**).[111]

KLF4-mediated gene transactivation is regulated on multiple levels by modulating KLF4's status through phosphorylation, acetylation, methylation, and ubiquitination in a context-dependent manner. KLF4 exerts many of its effects when the N-terminus of the molecule interacts with specific transcriptional co-activators, such as those in the p300-CBP coactivator family. KLF4 has also been shown to interact with the Cyclic adenosine monophosphate Response Element Binding (CREB)-protein. It also forms a complex with β-catenin to induce the telomerase promoter.

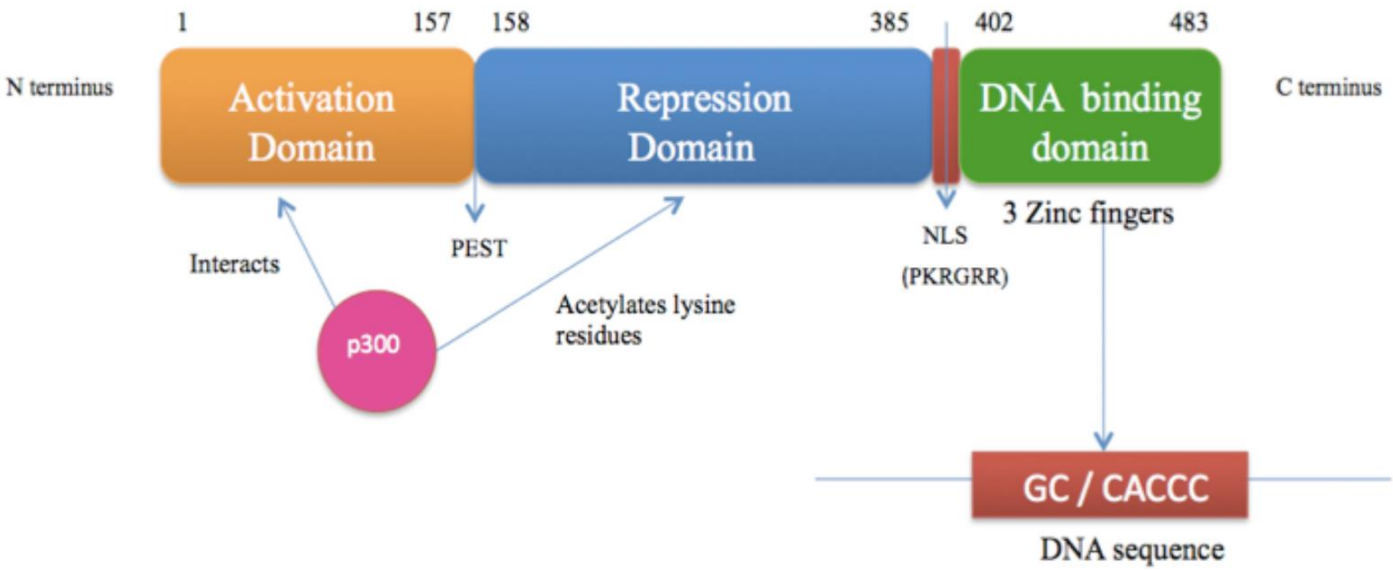


Figure G7.1. Schematic representation of the KLF4 gene.

KLF4 is involved in the regulation of cellular proliferation, differentiation, apoptosis, and somatic cell reprogramming. Particularly, *KLF4* is upregulated in non-dividing cells and its overexpression results in cell cycle arrest when the DNA is damaged. Indeed, a primary mechanism by which KLF4 regulates the cell cycle is by inducing the expression of Cyclin

Dependent Kinase Inhibitor 1A (*CDKN1A* - aka the gene encoding p21CIP1/WAF1, a CDK1 inhibitor).[112] This was elucidated by studies investigating the role of KLF4 in modulating cell cycle progression following DNA damage. By treating cultured cells with DNA-damaging agents, it was determined that KLF4 transactivates the CDKN1A promoter by binding to a specific SP1-like cis-element in the proximal region of the promoter. Subsequently, KLF4 recruits p53 to the CDKN1A promoter, allowing p53 to drive transcription of the *CDKN1A* gene.[113, 114] Activation of p21CIP1/WAF1 expression following DNA damage causes cell cycle arrest at both the G1/S and G2/M transition points. Moreover, KLF4 has been reported to inhibit expression of CCND1 and CCNB1, which promotes progression through the G1/S and G2/M boundary in the cell cycle.[115]

KLF4 is also important in maintaining genetic stability by regulating centrosome and chromosome number. [116] In the majority of tissues, KLF4 promotes cellular survival by suppressing the p53-dependent apoptotic pathway through direct inhibition of TP53 and suppression of BAX.[117, 118] Nevertheless, in certain types of cancer cell lines, activation of *KLF4* has been shown to promote apoptosis via the bcl2-bax pathway.[119]

7.2 The role of KLF4 in atherogenesis

KLF4 induces the activation of macrophages by regulating key inflammatory modulators such as NF-κB, TGF-β1, and inhibiting IL1β, while promoting the expression, translocation, and release of high-mobility group box 1.[120, 121] KLF4 is also constitutively produced in endothelial cells where its expression is driven by proinflammatory stimuli and shear stress. *KLF4* upregulation in endothelial cells induces the expression of multiple anti-inflammatory and anti-

thrombotic factors including endothelial nitric-oxide synthase and thrombomodulin, whereas its knockdown enhances TNFα-induced VCAM1. [122, 123]

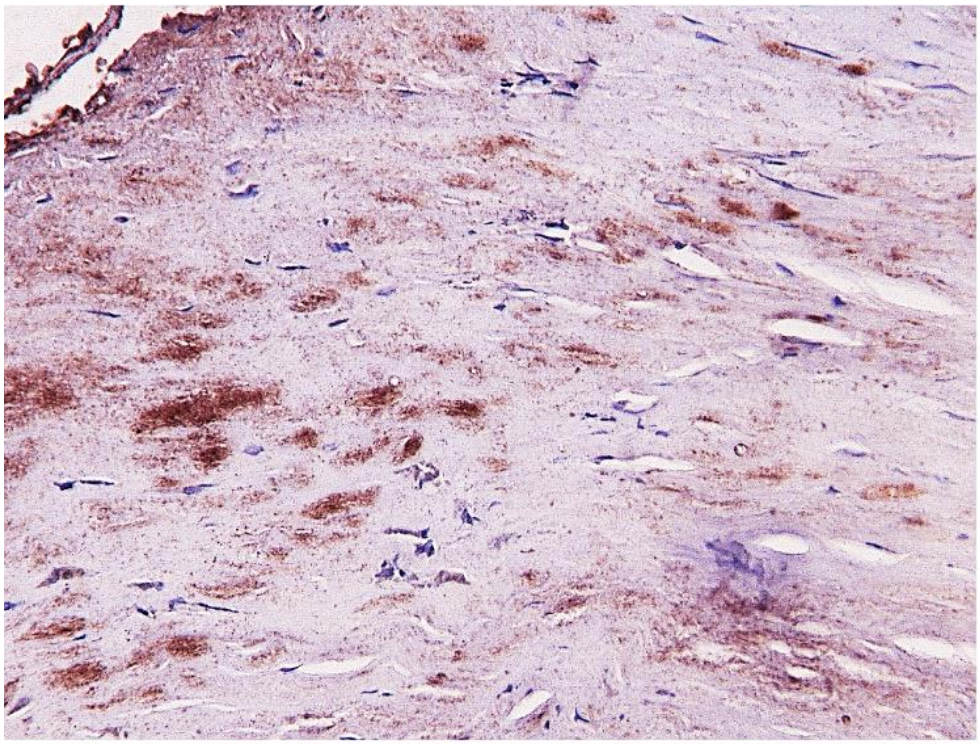


Figure G7.2. KLF4 immunohistochemical staining in macrophages cells (brown). Carotid artery sample from a patient with severe atheromatosis. Magnification ×400 *(adapted from Bakoyiannis C. and Mylonas K.S., et al., Increased Serum KLF4 in Severe Atheromatosis and Extensive Aneurysmal Disease. Ann Vasc Surg, 2020. 68: p. 338-343.)*

Normally, *KLF4* is not expressed in differentiated vascular smooth muscle cells in vivo. Yet, KLF4 is transiently induced following vascular injury (**Figure G7.2**).[124] In SMCs, *KLF4* has been identified as a transcriptional target of both bone morphogenetic proteins (BMP-2, -4 and -6) and TGF-β1 to modulate SMC differentiation.[125] Its transcriptional induction in SMC in response to BMP-4 and TGF-β1 is regulated by miRNA-143/145.[126] Functionally, KLF4 has a

critical role in maintaining the integrity of adherens junctions (AJs) and in preventing vascular leakage in response to inflammatory stimuli by regulating vascular endothelial (VE)-cadherin expression at the AJs. KLF4 also controls the acquisition of VE-cadherin-mediated endothelial barrier function. [127] Furthermore, KLF4 plays a key role in the regulation of gene transcription in the cardiovascular system. KLF4 is pivotal to the differentiation of cardiomyofibroblasts and to collagen synthesis by transcriptionally upregulating TGF-β1.[128]

7.3 Elevation of serum KLF4 levels in atheromatosis

Research from our group has shown that serum KLF4 is positively associated with the severity of atheromatosis (**Figure G7.3**).

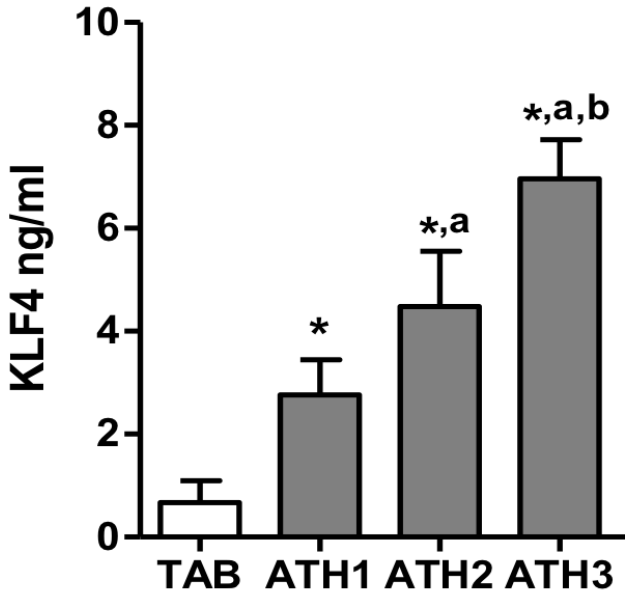


Figure G7.3: Serum KLF4 levels in subgroups. Data are presented as the mean ± SD. *p<0.05 between groups (ATHx) and TAB (control); a: p<0.05 between ATH1 and ATH2; b: p<0.05

between ATH2 and ATH3; c: p<0.05 between AA3 and AA1. *ATH1: moderate functional consequences [asymptomatic internal carotid stenosis >70% or claudication (Rutherford stage 3)]; ATH2: severe functional consequences [transient ischemic attack or rest pain (Rutherford stage 4)]; ATH3: permanent disability [stroke or tissue loss (Rutherford stage 5–6)]*.

Indeed, patients with permanent end-organ damage had higher serum KLF4 (6.96±0.75 pg/mL) compared to patients with asymptomatic internal carotid stenosis >70% or claudication (2.76±0.68 pg/mL, MD: -4.20; 95 % CI: -5.35 to -3.04, p<0.01) and to those with transient ischemic attack or rest pain (4.47±1.08 pg/mL, MD: -2.48; 95% CI: -3.76 to -1.21). Patients with moderate functional consequences also had higher serum KLF4 compared to patients experiencing milder atheromatosis sequalae (MD: -1.71; 95% CI: -2.80 to -0.62, p<0.01).[129]

CHAPTER 8

Bone-morphogenic protein 4 (BMP4)

8.1 BMP4 structure and function

BMP4 belongs to the bone morphogenic protein family which is a member of the TGF-β superfamily. *BMP4* is highly conserved evolutionarily and the coding gene is located on chromosome 14q22-q23.[130] The BMP4 peptide consists of 116 residues; seven of which are conserved and glycosylated. The monomers are held with disulphide bridges and three pairs of cysteine amino acids (cystine knot). BMP4 can form homodimers or heterodimers with similar BMPs (such as BMP7) to achieve superior ossifying activity.[131]

BMP4 dimers engage signaling by assembling heteromeric complexes of type I and II receptors on cellular surfaces. Type I (BMPR1) receptors are activin receptor-like kinases (ALKs). Type II (BMPR2) receptors include BMPR2, ACVR2A, ACVR2B, TGFBR2, and AMHR2. BMP4 preferentially binds ALK3 and 6 in complex with BMPR2, ACVR2A or ACVR2B.[132] Upon BMP4 binding, type II receptors phosphorylate and activate type I receptors, which in turn phosphorylate and activate R-SMADs. R-SMADs (Smad-1, Smad-5, Smad-8) form complexes with common SMAD4 to regulate transcription in the nucleus. In addition, BMP receptors can activate non-SMAD pathways such as ERK, JNK and p38 MAPK signaling pathways.[133] The

activation of the ERK and JNK pathways act to phosphorylate Smads and therefore regulate their activation. Furthermore, MAPK pathways may be able to directly affect Smad-interacting transcription factors via a JNK or p38 substrate thereby converging the two signaling pathways (**Figure G9.1**).

BMP4 signaling can be terminated by inhibitory SMADs including SMAD6 and SMAD7 (which are activated and induced by BMP signaling). For example, inhibitory SMADs can recruit SMURF1 and SMURF2 as well as E3 ubiquitin ligases to degrade activated receptors.[134] In addition, BMP signaling is regulated by different types of co-receptors (type III receptors) such as betaglycan and endoglin (which is highly expressed in cardiovascular tissues).[135] BMP signaling is also regulated by antagonists, such as Gremlin and Noggin, which bind to BMP2 and BMP4 to inhibit their action.[135] Similarly, matrix Gla protein can bind to and halt BMP2 and BMP4 signaling.[136]

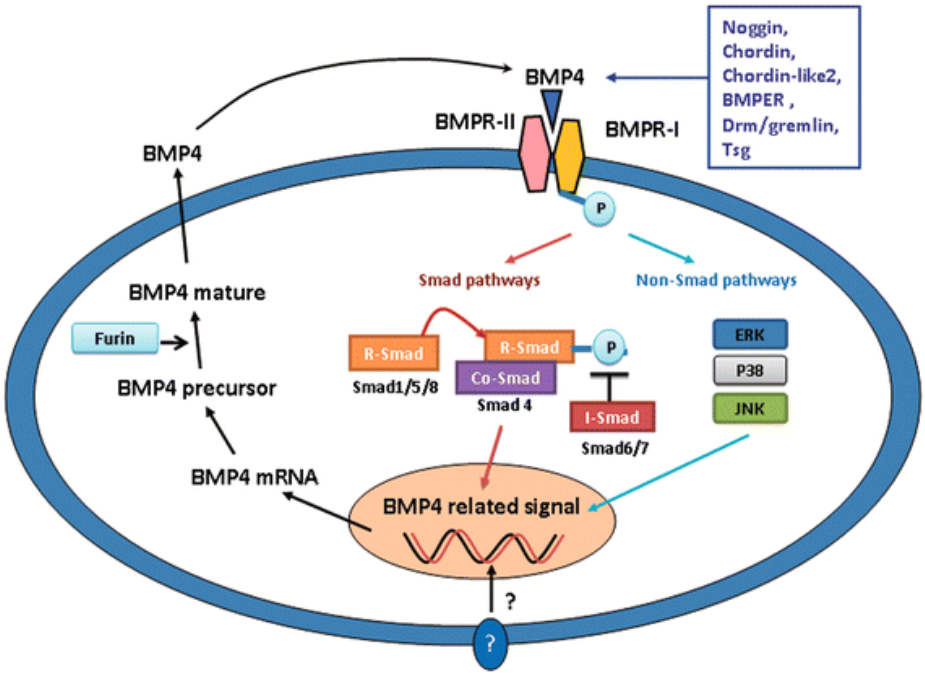


Figure G8.1. BMP4 signaling pathway *(adapted from Guo WT. et al. Heart Failure Reviews. 2014. v.19, p. 781–788.*

8.2 The physiologic role of BMP4

During embryogenesis, BMP4 is critical in the processes of early differentiation and dorsal-ventral axis establishment. BMP4 is central in endochondral ossification, muscle development, and bone mineralization. BMP4 activation has also been observed during the development of early mesoderm and germ cells. Limb bud regulation and development of the lungs, liver, ureteric bud, teeth facial mesenchyme, neural tube, and adipogenesis are also influenced by BMP4.[137]

BMP4 also contributes to angiogenesis, endothelial cell proliferation, and migration. Capillary tube formation is also increased upon activation of the BMP4 signaling pathway.[138] Furthermore, BMP4 is expressed by the endothelium in response to hypoxia. It also affects vascular SMC (VSMC) proliferation in a context-based fashion. For example, BMP4 inhibits SMC proliferation in the proximal pulmonary artery while promoting SMC cellular division in its distal aspect.[139]

8.3 The role of BMP4 in atherogenesis

BMP4 functions as a mechanosensitive gene and is induced by oscillatory shear stress. This results in the downstream upregulation of SMAD1/5/8.[140] BMP4 and other members of the TFGβ family act in various cell types including endothelial cells, SMCs, myofibroblasts, dendritic cells, T cells, monocytes and macrophages. They also recruit osteoblast-like and chondroblast-like cells to promote calcific remodeling and endochondral-like ossification.[141] In calcified atherosclerotic plaques, BMP4 signaling induces the expression of osteoblast and osteoclast regulatory proteins such as osteopontin, osteonectin, osteoprotegerin and receptor activator of

nuclear factor kappa-B ligand (RANKL).[142] BMP4 upregulation has also been associated with plaque vulnerability and instability.[143]

In VSMCs, BMP signaling promotes the expression of osteoblast lineage markers, such as alkaline phosphatase.[144, 145] Vascular SMCs may also express BMP2/4 to promote inflammation and monocyte infiltration via type II receptors. BMP4 promotes monocyte adhesion by inducing the expression of ICAM-1. BMP4 also favors the production of ROS via activating a nox1-based NADPH (nicotinamide adenine dinucleotide phosphate) oxidase.[146] BMP4 may also shift primordial pericytes towards an osteogenic phenotype.[147] Similarly, BMP4 promotes endothelial-to-mesenchymal transition.[148] Last but not least, BMP4 stimulation has been shown to inhibit the expression levels of the two most important cellular cholesterol transporters in macrophages (ABCA1 and ABCG1). This effect reduces cholesterol outflow thereby leading to foam cell formation.[149]

CHAPTER 9

SOX2

9.1 SOX2 structure

In 1990, Gubbay et al discovered a new transcription factor with a distinctive DNA-binding domain and correlated its function with testis determination. Since this coding sequence was located on the sex-determining region of the Y chromosome, it was termed sex-determining region Y (*SRY*) gene.[150] The SRY protein binds to specific DNA sequences with its high-mobility-group (HMG) domain. Notably, a novel family of developmental genes was established on the basis of sequence similarities to the HMG domain of the *SRY* gene. Particularly, the infamous Sry-related HMG box (SOX) proteins contain an HMG domain with at least 50% sequence similarity to the HMG domain of SRY. To date, 20 different *SOX* genes have been found in the murine and human genome which in turn have been divided into eight subgroups based on sequence identity and role.[151]

Among all *SOX* genes, *SOX2* is probably the most well studied one due to its key role in reprogramming somatic cells into iPSCs. [110] The human *SOX2* gene is located on chromosome 3q26.3–27 and encodes a protein of 317 amino acids.[152] The epicenter of SOX2 is its HMG domain which remains highly conserved among species. In addition to binding to specific DNA

consensus sequences, this domain also contains a nuclear localization and a nuclear export signal. The function of the C-terminal transactivation domain is to recognize and bind to the promoters of target genes thereby inducing or repressing gene expression in a context-dependent fashion.

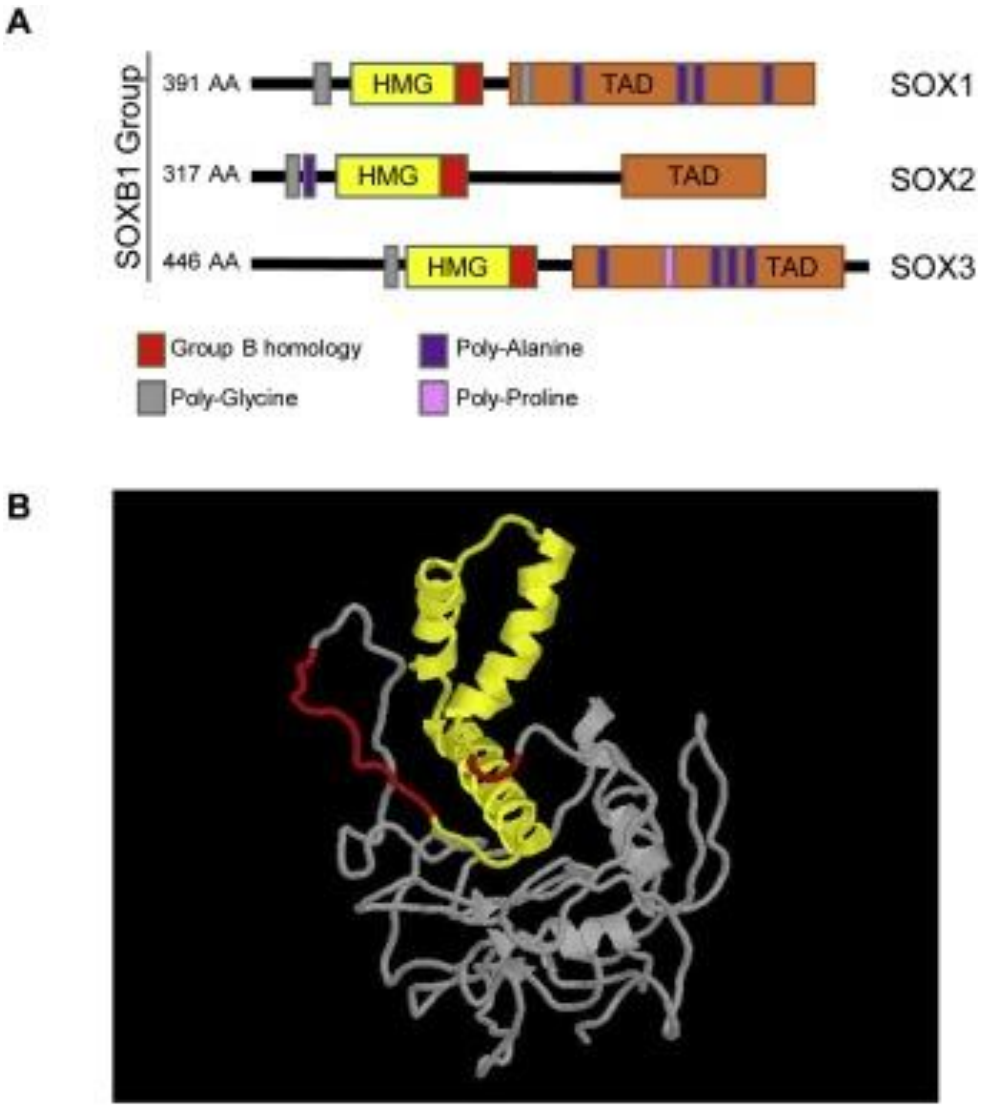


Figure G9.1. Schematic drawing and crystal structure of SOX2. A: SOX1, SOX2 and SOX3 belong to the SOXB1 subgroup. All family members have the DNA-binding HMG domain in common. B: Crystal structure of SOX2 modulated with RasMol. The yellow and red part is reconstructed based on NMR results while the grey part is a theoretical model based on IntFOLD

calculations with a global model quality score of 0.3263. The HMG domain is labeled in yellow. (HMG: High-mobility-group box DNA-binding domain; TAD: Transactivation domain), *(adapted from Novak D., Hüser L., Elton J.J., Umansky V., Altevogt P., and Utikal J., SOX2 in development and cancer biology. Semin Cancer Biol, 2020. 67(Pt 1): p. 74-82.)*

9.2 SOX2 function

LIF signaling maintains pluripotency in embryonic stem cells by activating *SOX2* downstream of the JAK-STAT pathway. OCT4 (Octamer-binding transcription factor 4), SOX2 and NANOG regulate transcription of all pluripotency circuitry proteins in the LIF pathway.[153]

Nucleophosmin 1, a transcriptional regulator involved in cellular division, individually forms complexes with SOX2, OCT4 and NANOG in embryonic stem cells.[154] SOX2 forms a trimeric complex with OCT4 on DNA and controls the expression of a number of genes involved in embryonic development such as *YES1, FGF4, UTF1* and *ZFP206*. It also binds to the proximal enhancer region of *NANOG*. Surprisingly, regulation of *OCT4-SOX2* enhancers can occur even in the absence of SOX2, likely due to expression of other SOX proteins. That said, the primary role of SOX2 in embryonic stem cells is to control OCT4 expression. Importantly, SOX2 and OCT4 synergistically perpetuate their own expression when expressed concurrently.[155]

As previously mentioned, SOX2 in conjunction with OCT4, c-MYC and KLF4 can produce iPSCs (Yamanaka factors). [110] Loss of pluripotency is achieved by hypermethylation of certain SOX2 and OCT4 binding sites in male germ cells [156] as well as by post-transcriptional suppression of SOX2 by miR134.[157] *SOX2* is expressed at the two-cell stage of embryogenesis with increasing levels up to the blastocyst stage. Indeed, *SOX2* plays an essential role in the

emergence of the pluripotent inner cell mass during early embryonic development and its absence results in embryonic lethality. While *SOX2* is homogeneously expressed in the inner cell mass, it is downregulated in certain cell populations when the embryo undergoes the transformation from a single-layered to a multi-layered structure during gastrulation. Subsequent *SOX2* upregulation characterizes primordial germ cells, gut endoderm, presumptive neuroectoderm, sensory placodes and pharyngeal arches. The specification of the neural lineage in early embryonic development strongly relies on the activity of *SOX2* which exerts its effect by antagonizing transcription factors that would favor the development of non-neural cell lineages.[158, 159]

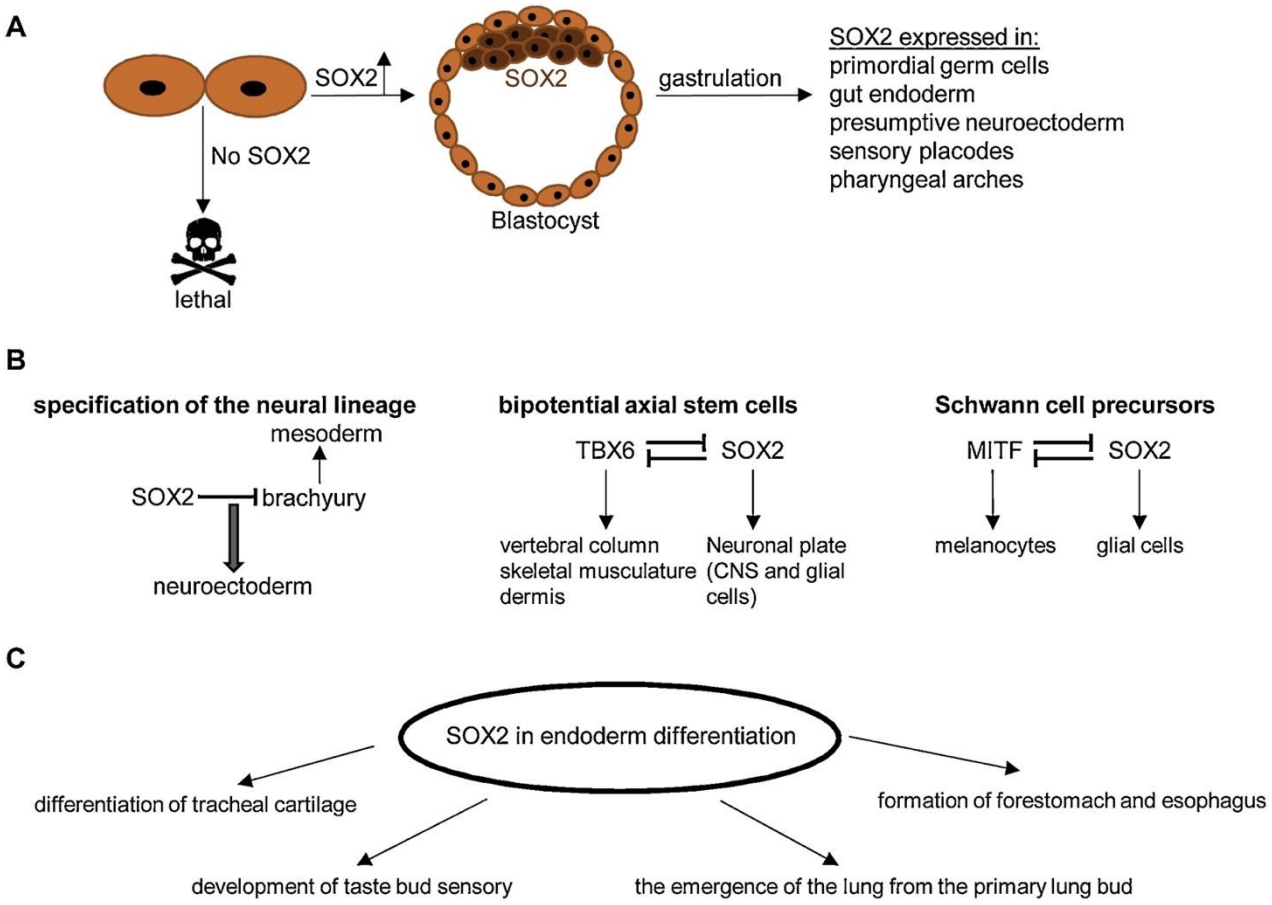


Figure G91. Effects of SOX2 on embryogenesis. TBX6: T-Box Transcription Factor 6, MITF: Melanocyte Inducing Transcription Factor, CNS: central nervous system *(adapted from Novak D.,*

Hüser L., Elton J.J., Umansky V., Altevogt P., and Utikal J., SOX2 in development and cancer biology. Semin Cancer Biol, 2020. 67(Pt 1): p. 74-82.

9.3 The role of SOX2 in atherogenesis

Endothelial-mesenchymal transition contributes to vascular disease.[160, 161] Indeed, endothelial SOX2 has been shown to upregulate in calcified atherosclerotic aortas of fat-fed *ApoE*$^{-/-}$ mice. On the other hand, in the same animal mode, SOX2 depletion via shRNA decreased both stem cell marker expression and lesion calcification in fat-fed ApoE−/− mice. That said, there was no significant change in serum lipid levels or size of atherosclerotic lesions, as measured in the aortic sinuses.[160]

CHAPTER 10

OCT4

10.1 OCT4 structure and function

OCT4 is a transcription factor that is encoded by the *Pou5f1* gene (chromosome 6p21.33). It contains an octamer motif with a highly conserved DNA sequence (AGTCAAAT) that binds to target genes and controls their expression pattern. During fetal development, OCT4 plays a vital role in determining the fates of both inner mass cells and embryonic stem cells (ESCs) and has the ability to maintain pluripotency throughout embryogenesis.[10]

As previously mentioned, OCT4 is one of the Yamanaka reprogramming factors that can produce iPSCs.[162] It forms a trimeric complex with SOX2 (or SOX15) on DNA and controls the expression of a number of genes involved in embryonic development such as *YES1, FGF4, UTF1* and *ZFP206*. OCT4 contributes to the rapid cell cycle of ESCs by promoting progression through the G1 phase, specifically via transcriptional inhibition of cyclin-dependent kinase inhibitors such as p21. *OCT4* upregulation has also been identified in several types of malignancies, including pancreatic, lung, liver and testicular germ cell tumors.[163]

10.2 The role of OCT4 in atherogenesis

OCT4 is typically deactivated in normal adult somatic cells. Although limited, there is evidence suggesting that OCT4 expression varies during the development of atheromatosis. During the early phases of proatherogenic inflammation, *OCT4* appears to be upregulated in SMCs and SMC-derived macrophage cells of the arterial wall. Nevertheless, prolonged atherogenic stimuli led to *OCT4* loss in over 80% of $Apoe^{-/-}$ mice who were exposed to Western diet for three weeks. Downregulation of OCT4 appears to favor plaque development in several ways.[164]

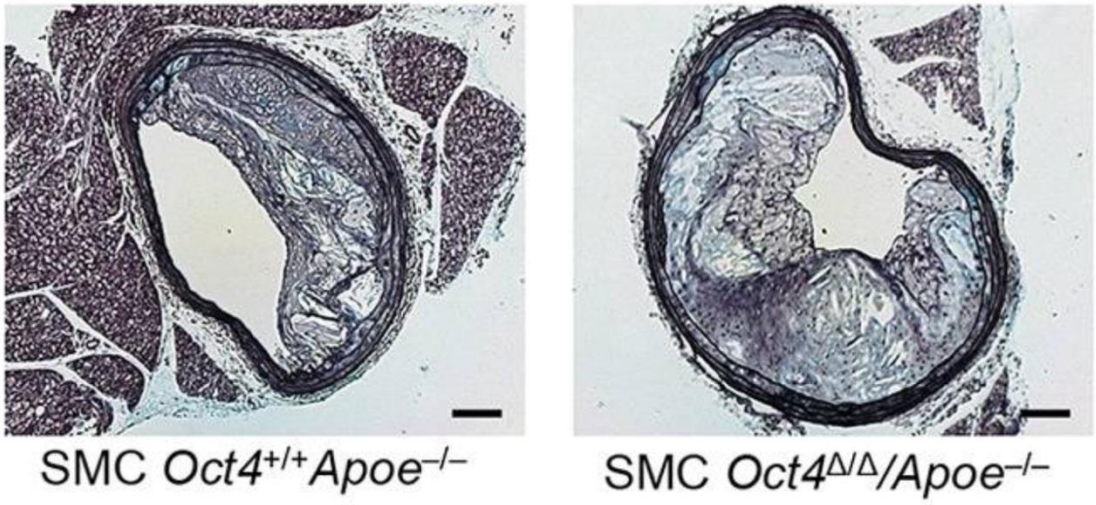


Figure G10.1 Movat staining of a BCA from a SMC $Oct4^{+/+}Apoe^{-/-}$ and SMC $Oct4^{\Delta/\Delta}Apoe^{-/-}$ mouse. Scale bar, 50 μm *(adapted from Cherepanova O.A. et al., Activation of the pluripotency factor OCT4 in smooth muscle cells is atheroprotective. Nat Med, 2016. 22(6): p. 657-665.)*

Loss of *OCT4* within SMCs has been shown to result in a marked reduction in the number of SMC-derived cells within atherosclerotic lesions (including within the fibrous cap) due to the downregulation of several OCT4-mediated migratory genes such as *Limch1, Slit3, Kcnd3, Sorl1, Cacna1c, Cap2, Lgr6*. These changes impair SMC migration from the media to the intima. That

said, no changes in apoptosis or proliferation rates have been described to date. Paradoxically, the reduction of SMC-lineage cells is associated with an increase in overall lesion size as a result of large increases in non-cellular components of the lesion, including lipids, hemorrhagic products, and necrotic tissue.[165] Indeed, SMC *Oct4*$^{\Delta/\Delta}$*Apoe*$^{-/-}$ mice exhibited a large increase in branchiocephalic artery (BCA) lesion size area within the external elastic lamina, and area within the internal elastic lamina, as well as reduced lumen size compared to SMC *Oct4*$^{+/+}$*Apoe*$^{-/-}$ littermate control mice (**Figure G10.1**).

Moreover, SMC-specific knockout of *OCT4* was associated with increases in multiple indices of plaque instability including increased necrotic core area, decreased cell density in the non-necrotic lesion areas, reduced collagen maturation based on PicroSirius Red staining, as well as increased lipid accumulation based on Oil Red O staining (**Figure G10.2**) and increased intra-plaque hemorrhage based on TER119 staining.[165]

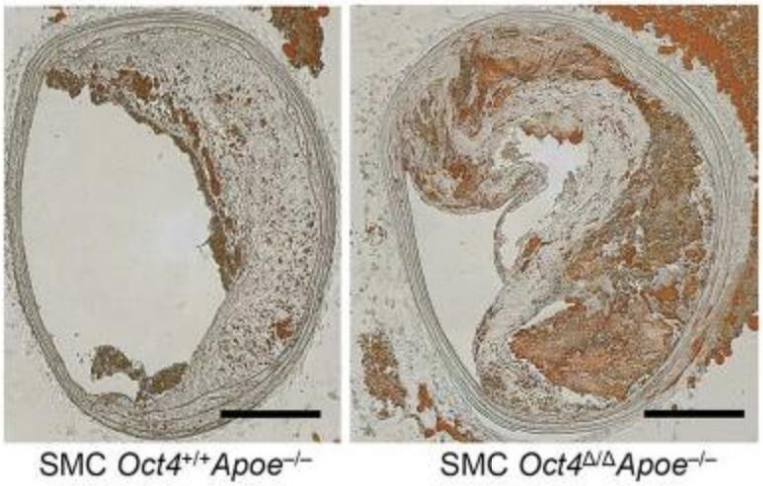


Figure G10.2 Oil Red O staining of representative BCA sections of SMC *YFP*$^{+/+}$*Oct4*$^{+/+}$*Apoe*$^{-/-}$ and SMC *YFP*$^{+/+}$*Oct4*$^{\Delta/\Delta}$*Apoe*$^{-/-}$ mice *(adapted from Cherepanova O.A. et al., Activation of the pluripotency factor OCT4 in smooth muscle cells is atheroprotective. Nat Med, 2016. 22(6): p. 657-665.)*

CHAPTER 11

NANOG

11.1 NANOG structure

The homeobox protein NANOG is a transcriptional factor that helps ESCs maintain pluripotency by suppressing cell determination factors. The *NANOG* gene encompasses 8,265 base pairs (bp) of DNA in humans and is located on chromosome 12p13.31. A 299 bp *NANOG* promoter region (-264 to +35) upstream of exon 1 contains five CpG-dinucleotides, which are subjected to DNA-methylation. Furthermore, this *NANOG* promoter region contains an OCT3/4-SOX2 binding motif, a TATA-box and binding sites for the transcription factors AP-2, SP1 and TFIID. There are also binding sites for AP-1, SP1 and Oct3/4. (**Figure G11.1**).[166]

Figure G11.1 Structure of the NANOG gene *(adapted from Schorle H. et al. Atlas of Genetics and Cytogenetics in Oncology and Hematology. 2012)*

The predominant isoform of the NANOG protein (aka NANOG1) consists of 305 amino acids and has a molecular weight of 34.6 kilo Daltons. It includes a Serine-, Threonine- and Proline-rich N-terminal region as well as eight W-repeats at its C-terminus. The DNA-binding homeodomain motif spans from amino acid 95 to 155. Formation of secondary structures (helix, strand and turn) occurs mainly within the homeobox-coding region (**Figure G11.2)**.[166]

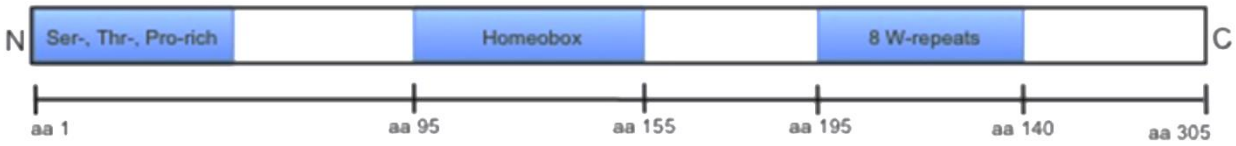


Figure G11.2 Structure of the NANOG protein *(adapted from Schorle H. et al. Atlas of Genetics and Cytogenetics in Oncology and Hematology. 2012*

11.2 NANOG regulation

Physiologically, *NANOG* expression is restricted to pluripotent cells and downregulates upon differentiation.[167] The *NANOG* gene is transcribed under the control of a regulatory region that lies within 332 bp upstream of the transcriptional start site. This region contains highly conserved Octamer and SOX elements. Importantly, OCT4 and SOX2 are the major transcription factors that bind to the *NANOG* promoter to induce transcription.

Additional pluripotent factors that can also activate *NANOG* expression include Forkhead Box Protein-D3 and LIF-STAT3. Catenin-β is involved in the upregulation of *NANOG* through binding with OCT3/4 complexes in the nucleus. OCT4 can also bind to a novel PSBP (Pluripotential cell-specific SOX element-Binding Protein) leading to *NANOG* expression. OCT4 mainly binds to the Octamer element, while PSBP preferentially interacts with the SOX element.[168]

When expressed, NANOG blocks differentiation. Therefore, negative regulation of NANOG is required to promote differentiation during embryonic development. The tumor suppressor protein p53 binds to the *NANOG* promoter to inhibit its transcription. Other factors that can inhibit NANOG expression include transcription factor-3 of the WNT pathway, GRB2 (Growth Factor Receptor-Bound Protein-2)/SOS/Ras/ERK (Extracellular Signal-Regulated Kinase). Last but not least, NANOG is able to auto-repress its own expression in differentiating cells via binding to its own promoter following interaction with ZNF281/ZFP281. This leads to recruitment of the NuRD complex and subsequent repression of *NANOG* expression.[167]

11.3 NANOG effects

NANOG regulates stem cell pluripotency by assuming a bifunctional role to repress genes important for differentiation whilst activating the ones necessary for self-renewal.[169] First, NANOG interacts with SOX2 to regulate the expression of *REX1/ZFP42* by binding to its promoter. Second, NANOG represses GATA6, which results in repression of *GATA4*, thereby inhibiting ESC mesodermal differentiation. Third, NANOG regulates the expression of *OCT4* and *SOX2* directly and works with them to establish ESC identity. These three genes also repress

Caudal-type homeodomain transcription factor Cdx2 expression which is necessary for trophectoderm development. NANOG also blocks BMP-induced mesoderm differentiation by physically interacting with SMAD1 thereby interfering with the recruitment of coactivators to the active SMAD transcriptional complexes (**Figure G11.3**).[169] Of note, NANOG (particularly its isoform 8) seems to be upregulated in a variety of hematologic and solid organ malignancies.[170]

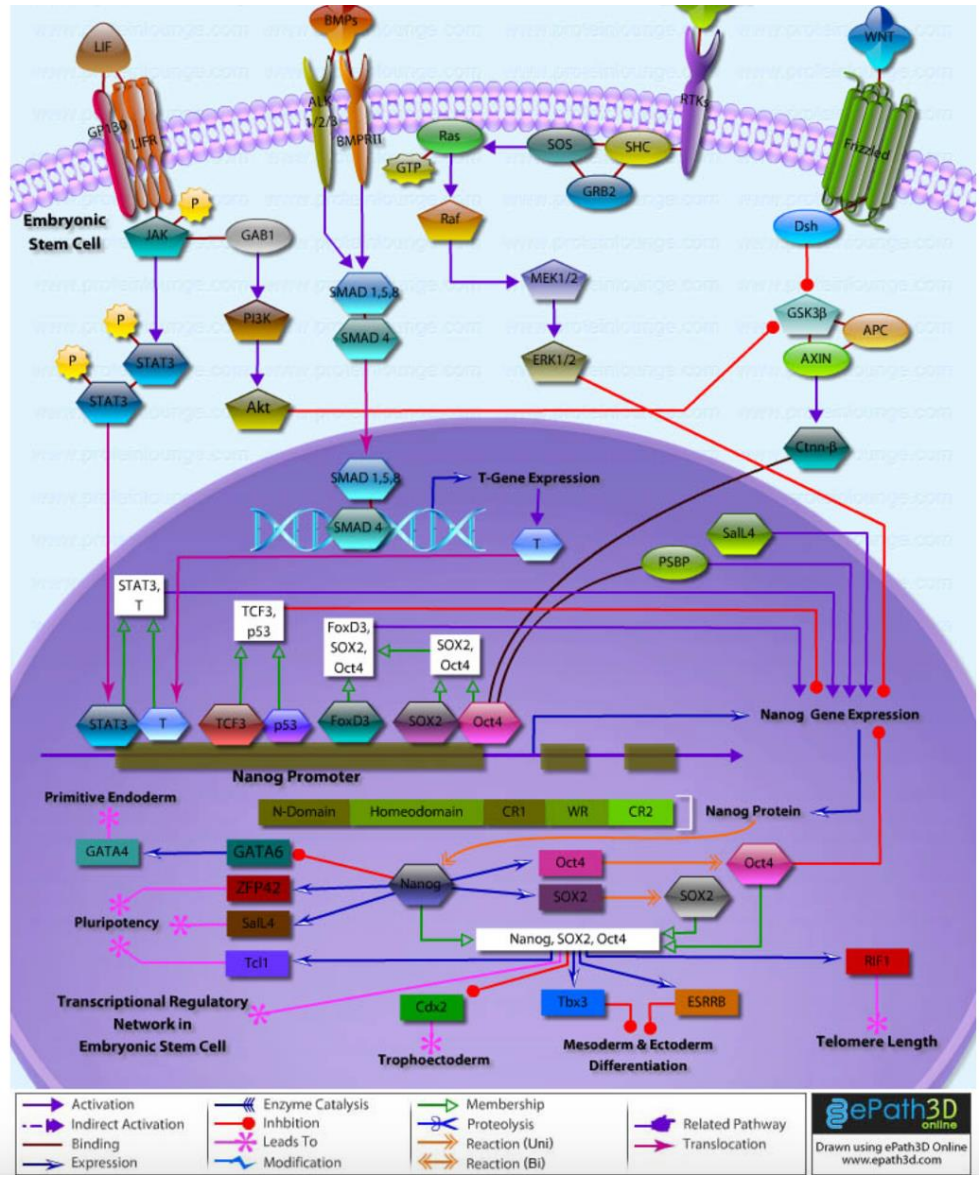


Figure G11.3 Complex genetic network orchestrated by NANOG *(modified reproduction from Abeomics, Inc. San Diego, California, USA)*

11.4 NANOG in cardiovascular disease

The role of NANOG in atherogenesis has not been thoroughly assessed to date. Nevertheless, scarce data exist regarding its impact on acute aortic dissection. Indeed, NANOG seems to be highly expressed in the dissected aortic wall which leads to osteopontin upregulation and ultimately VSMC phenotype switch. Specifically, overexpression of *NANOG* in SMCs induces the upregulation of synthetic marker MMP 2 and attenuates the expression of contractile markers such as α-smooth muscle actin and smooth muscle 22α. Overexpression of *NANOG* also enhances the proliferation, migration, and anti-apoptosis capabilities of vascular SMCs.[171]

Furthermore, *NANOG* upregulation in response to vascular injury has been shown to induce the loss of VE-cadherin from adherens junctions. Further sequalae include endothelial cell proliferation accompanied by asymmetric cell division which form cellular aggregates in hanging drop assays indicating the acquisition of a de-differentiated phenotype. [172]

CHAPTER 12

NOTCH1

12.1 NOTCH1 structure and canonical signaling

NOTCH1 is located on chromosome 9q34.3 and constitutes one of the four genes encoding the NOTCH pathway. NOTCH signaling is an evolutionarily conserved intercellular signaling pathway that regulates interactions between physically adjacent cells through binding of NOTCH family receptors to their cognate ligands.[173]

The NOTCH1 preproprotein is proteolytically processed by a furin-like convertase in the trans-Golgi network to yield an active, ligand-accessible form. Its cleavage results in C- and N-terminal fragments. Particularly, NOTCH proteins are characterized by N-terminal Epidermal growth factor (EGF)-like repeats followed by LIN-12/notch repeat (LNR) domains. [173, 174]

Following post-translational modification, two polypeptide chains heterodimerize to form the mature Type I transmembrane receptors on the cell surface. NOTCH1 determines cellular fate by acting as a receptor for membrane-bound ligands Jagged-1 (JAG1), Jagged-2 (JAG2) and Delta-1 (DLL1). Following ligand binding, NOTCH1 is cleaved by ADAM17 to yield a membrane-associated intermediate fragment called notch extracellular truncation (NEXT).[174]

Following its endocytosis, NEXT is cleaved by one of the catalytic subunits of γ-secretase to release a NOTCH-derived peptide containing the intracellular domain (NICD). The released NICD forms a transcriptional activator complex with RBPJ/RBPSUH which is a constitutive repressor of NOTCH signaling. RBPJ represses NOTCH target gene expression by recruiting a co-repressor complex, which includes NCOR1, NCOR2, SNW1, CIR, HDAC1, HDAC2, SPEN and FHL1 and SAP30. NICD binding to RBPJ replaces the co-repressor complex with a co-activator complex which includes MAML1-3, EP300 and SNW1. Primary NOTCH target genes include two families of transcriptional factors Hes (including HES1 and HES5) as well as Hey (including HEY1 and HEY2). Other NOTCH1 target genes include *CCND1*, *CDKN1A*, *GATA3* and *PTCRA*.[175]

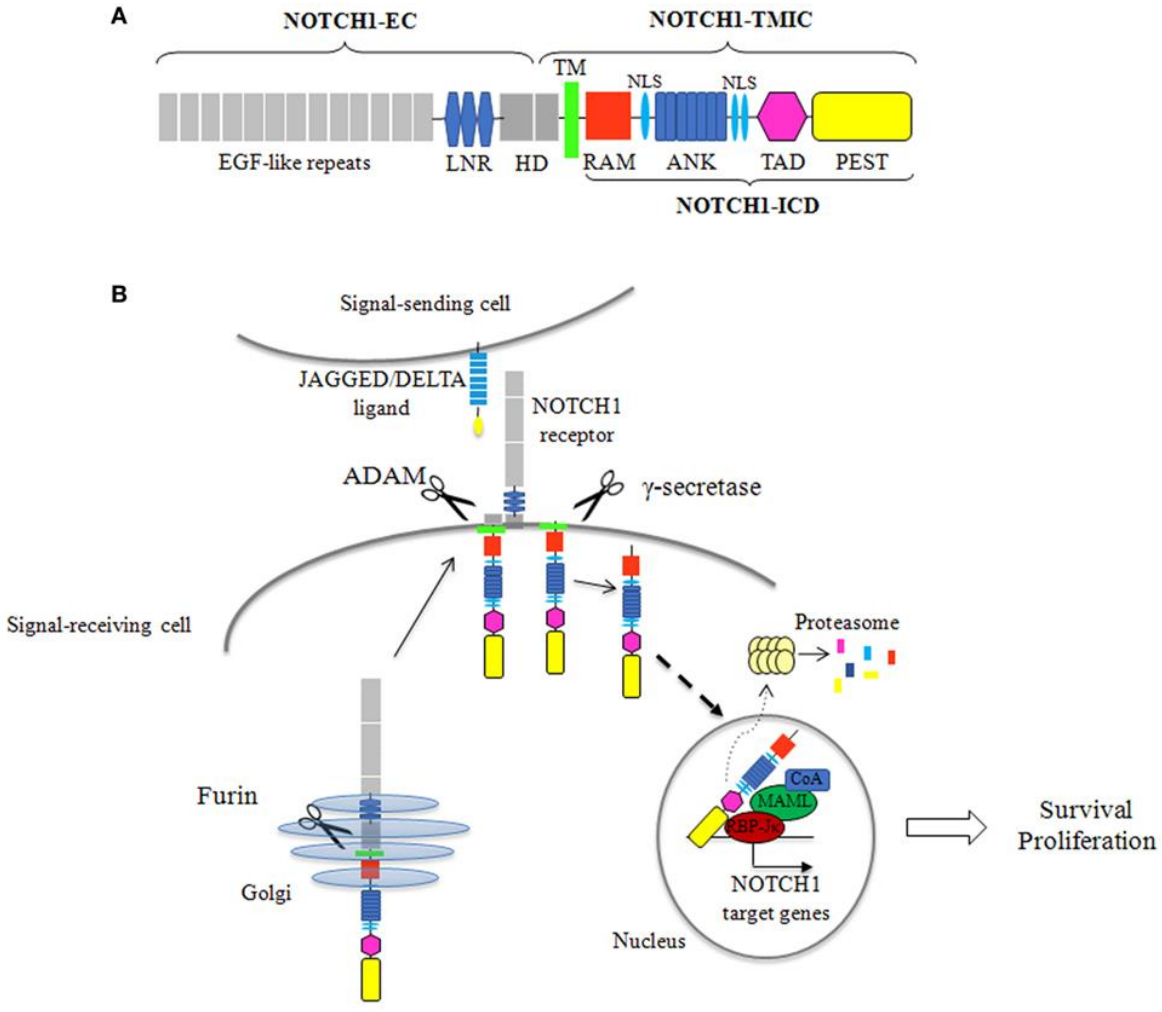


Figure G12.1 NOTCH1 formation and post-translational modifications *(adapted from Rosati E. Frontiers in Oncology, 27 Jun 2018, 8:22)*

12.2 NOTCH1 non-canonical pathways

In addition to conventional NOTCH signaling, there is increasing evidence showing RBPJ independent non-canonical pathways.[176] Interaction of NOTCH-1 with LCK- PI3K may mediate non-nuclear crosstalk with AKT. Notch stimulation through AKT pathway leads to downregulation of MYC expression. Activation of the SRC/STAT3 pathway by NOTCH signaling is dependent on the expression of the HES1 transcription factor which enhances SRC phosphorylation.

The activated SRC kinase has been found to be responsible for the enhanced phosphorylation of STAT3. The HES1 and HES5 proteins associate with and promote the complex formation between JAK2 and STAT3, thereby achieving STAT3 phosphorylation and activation. Ultimately, activated STAT3 translocates from the cytoplasm to the nucleus and induces transcriptional activation of several target genes, including *HIF1A*.[176]

12.3 NOTCH1 function

The NOTCH signaling pathway is involved in processes related to cell fate specification, differentiation, proliferation, and survival. In the cardiovascular system, NOTCH signaling controls endothelial cell proliferation, endothelial cell survival, arterial versus venous specification

and tip cell versus stalk cell specification during sprouting angiogenesis.[177-179] NOTCH signaling also regulates endothelial-to-mesenchymal and endothelial-to-hematopoietic transition during embryonic development.[178]

NOTCH1 is involved in the maturation of CD4(+) and CD8(+) cells in the thymus. It is also a central driver of neurogenesis. Importantly, during cerebellar development, NOTCH1 is involved in the differentiation of Bergmann glia. Furthermore, it represses neuronal and myogenic differentiation.[180] Mutations in *NOTCH1* are associated with several conditions including bicuspid aortic valve disease, Adams-Oliver syndrome, T-cell acute lymphoblastic leukemia, chronic lymphocytic leukemia, head and neck squamous cell carcinoma as well as various other types of malignancies.[181]

12.4 The role of NOTCH1 in atherogenesis

Anecdotal data suggested that *NOTCH1* haploinsufficiency may increase aortic atherosclerosis.[182] Subsequent research confirmed that *NOTCH1* is flow-responsive and involved in regulating endothelial inflammatory genes.[183] Indeed, *NOTCH1* is sensitive to shear stress and appears to be essential for the maintenance of junctional integrity, cell elongation, and suppression of proliferation. Of note, NOTCH signaling scales with the magnitude of fluid shear stress. Specifically, only laminar flow increases the expression of *NOTCH1*. Conversely, low shear stress leads to downregulation of *NOTCH1*. This destabilizes cellular junctions and triggers endothelial proliferation (**Figure G11.2**).

Under normal circumstances, *NOTCH1* is activated by cell–cell contact to promote cell cycle arrest.[184] Reduced NOTCH1 levels favor the overexpression of cyclins and CDKs as well

as of several additional regulators of cell cycle. As a result, in the absence of NOTCH1, cellular proliferation spikes. EFNB2, a marker of arterial identity, is also downregulated when NOTCH1 is suppressed. It should be emphasized that *NOTCH1* suppression results in reduced inter-cellular stability via upregulation of genes involved in intracellular calcium homeostasis. These include CAMK2B (a calcium/calmodulin-dependent protein kinase) and Apelin. Both of these molecules are involved in regulation of calcium release from the endoplasmic reticulum via SERCA and ryanodine receptors.[185, 186] Connexin-37 and aconitase levels are also increased in the absence of NOTCH1 - which in turns leads to deficiencies in the formation of gap junctions.[187]

Lastly, NOTCH1 directly inhibits the expression of pro-inflammatory cytokines including CXCL2, ICAM1, CXCR4, and several interleukins. Therefore, downregulation of *NOTCH1* promotes pro-atherogenic inflammation on a molecular level. Conversely, TNF-α, IL1β, and oxidized lipids have been shown to inhibit the expression of endothelial *NOTCH1*.[182] Furthermore, binding of monocytes and macrophages is also likely facilitated by intercellular gaps, since accumulation of fibrin enables attachment and further spreading of inflammatory cells.

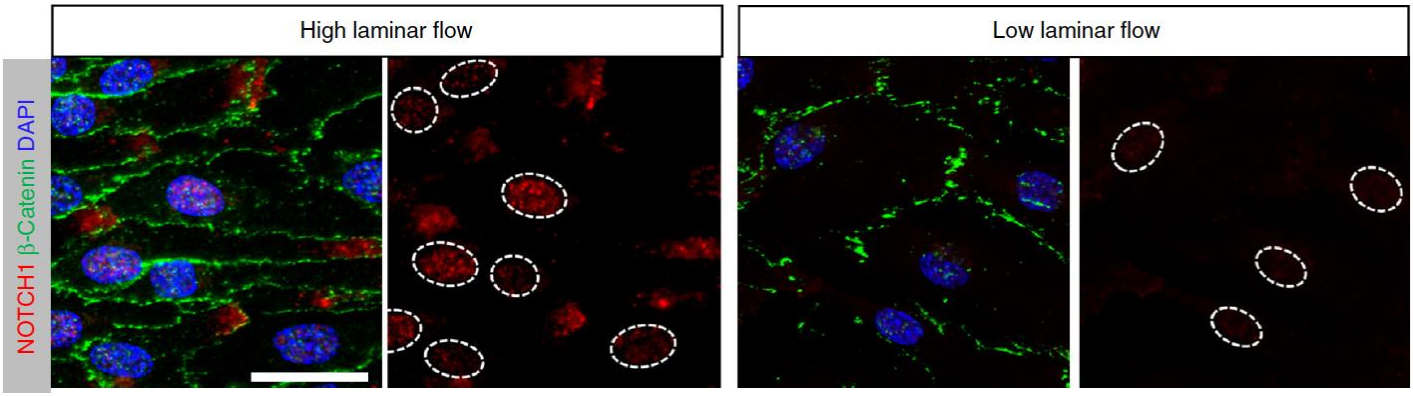


Figure G12.2 NOTCH1 expression in decreased in the setting of low shear stress *(adapted from Mack J.J., et al., NOTCH1 is a mechanosensor in adult arteries. Nat Commun, 2017. 8(1): p. 1620)*

CHAPTER 13

HIF1α

13.1 HIF1a structure

The hypoxia-inducible factor 1a (*HIF1a*) gene is located on chromosome 14q23.2 and encodes the alpha subunit of the mature HIF1 transcription factor.[188] Under normoxic conditions, the *HIF1A* gene is constitutively expressed in low levels, whereas in the setting of hypoxia, *HIF1A* transcription is strongly upregulated.[189] HIF1A contains a basic helix-loop-helix domain near the C-terminal, followed by two distinct PAS (PER-ARNT-SIM) domains, and a PAC (PAS-associated C-terminal) domain.[190] The HIF1A polypeptide also contains a nuclear localization signal motif, two transactivating domains CTAD and NTAD, and an intervening inhibitory domain that can halt the transcriptional activities of CTAD and NTAD. A total of three HIF1A isoforms can be produced by alternative splicing. Nevertheless, isoform 1 is the most commonly produced and extensively studied variant.[188]

The mature HIF1 protein is characterized by a heterodimeric basic helix-loop-helix structure that is composed of HIF1A (alpha subunit) and the aryl hydrocarbon receptor nuclear translocator (ARNT) (beta subunit). This composite heterodimer binds core DNA sequence 5'-

TACGTG-3' within the hypoxia response element (HRE) of target gene promoters. Of note, the discovery of HIF won the Nobel Prize in Physiology or Medicine 2019.

13.2 HIF1a function

HIF1 is known to induce transcription of more than 60 genes in response to hypoxia, including vascular endothelial growth factor (VEGF) and erythropoietin which promote angiogenesis and erythropoiesis. HIF1 also induces transcription of genes involved in cell proliferation and survival, as well as glucose and iron metabolism.

HIF1A activity is regulated by a variety of post-translational modifications including hydroxylation, acetylation, and phosphorylation. HIF1A stability, subcellular localization, as well as transcriptional activity are directly affected by oxygen levels.[191] Under normoxic conditions, the von Hippel-Lindau (VHL)-mediated ubiquitin protease rapidly degrades HIF1A.[192] On the other hand, under hypoxia, HIF1A protein degradation is prevented and HIF1A levels accumulate to associate with HIF1B to form the mature HIF1 transcription factor. Activation requires recruitment of transcriptional coactivators such as CREBBP and EP300.[193]

Enzymes prolyl hydroxylase (PHD) and HIF prolyl hydroxylase (HPH) are involved in post-translational modification of HIF1A proline residues (P402 and P564 within the ODD domain). This enables VHL association with HIF1A.[194] PHD is dependent on oxygen levels as it requires oxygen as one of its main substrates to transfer to the proline residue of HIF1A. The hydroxylated proline residue of HIF1A is then recognized and buried in the hydrophobic core of VHL, which itself is part of a ubiquitin ligase enzyme.[192] The hydroxylation of HIF1A proline residue also regulates its ability to associate with co-activators under hypoxia. Overall, HIF1 plays

an essential role in embryonic vascularization, tumor angiogenesis and pathophysiology of ischemic disease.[195] In monocytes, it also induces expression of angiotensin-converting enzyme 2 (ACE2) and cytokines such as IL1B, TNF, IL6, and interferons.

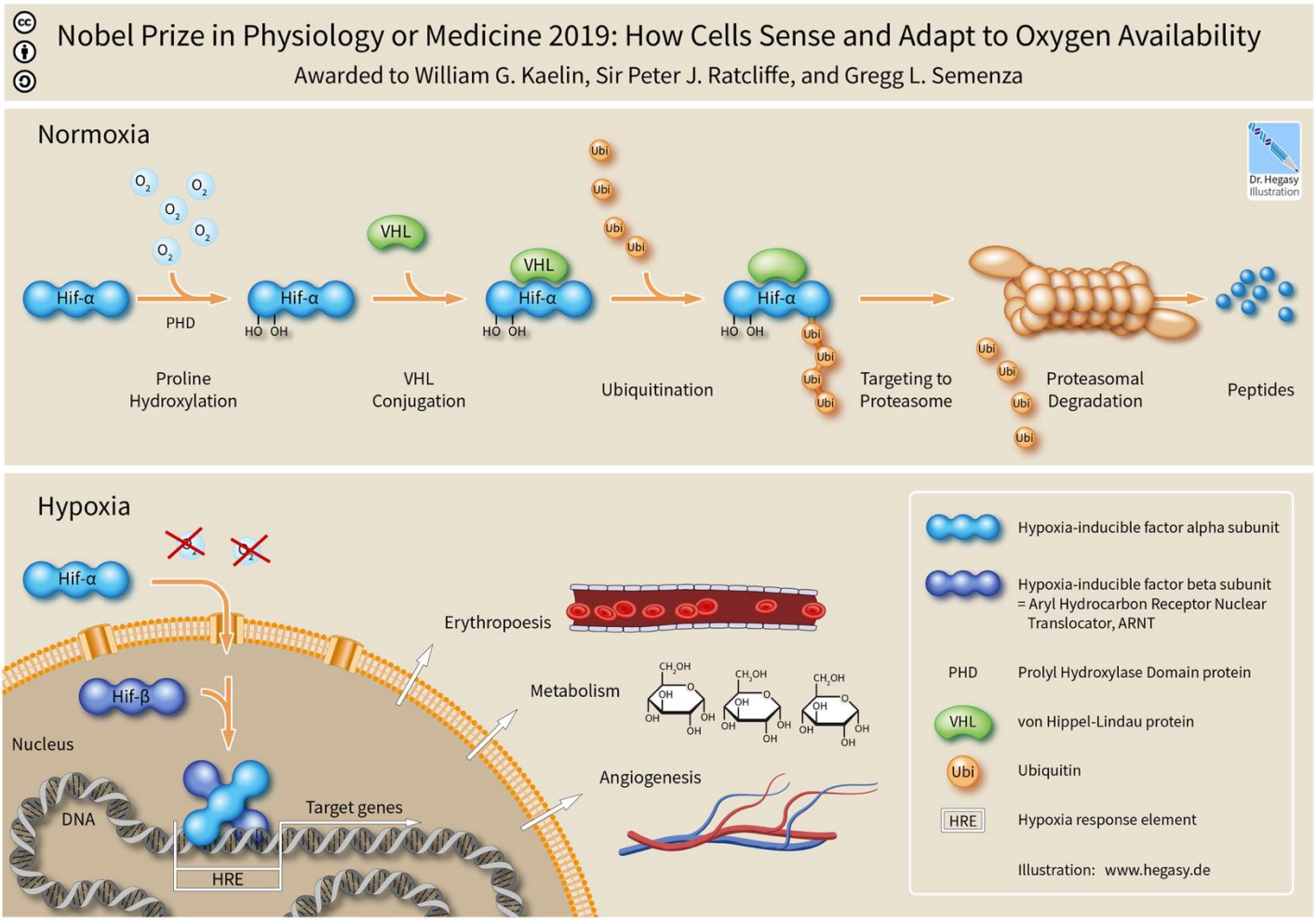


Figure G13.1 HIF1a cycle in response to oxygen levels *(Illustration by www.hegasy.de)*

13.3 The role of HIF1a in atherogenesis

Although HIF1α is physiologically upregulated in response to hypoxia, low shear stress can also activate HIF1α even under normoxic conditions via two parallel pathways. First, low

shear stress in atheroprone regions induces the expression of NF-κB which directly leads to overexpression of the *HIF1a* gene. Second, both in vitro and in vivo models have shown that Cezanne levels increase in response to low hemodynamic shear stress. This is an enzyme that removes ubiquitin moieties from target proteins to prevent proteasomal degradation. Ultimately, Cezanne-mediated de-ubiquitination rescues HIF1a from VHL-mediated degradation thereby sustaining high intracellular levels of the mature HIF1 heterodimeric molecule.[196] *HIF1a* is also upregulated in atheroprone regions due to localized endothelial hypoxia which may be caused by secondary flows that sway oxygen away from the arterial wall or from oxygen utilization by metabolically active macrophages.[197]

HIF1α drives atherosclerotic plaque progression by promoting plaque formation and intraplaque angiogenesis.[198] *HIF1α* upregulation in endothelial cells has been shown to enhance the expression of proinflammatory microRNA-19a which in turn induces CXCL1 expression and monocyte adhesion.[199] Additional adhesion molecules such as ICAM1 and VCAM1 are also upregulated. Furthermore, HIF1α drives inflammation and cellular proliferation in low shear stress conditions by inducing the expression of several enzymes involved in the metabolism of glucose. These include the glycolysis regulator 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3), glycolysis enzymes (hexokinase 2 & enolase 2), as well as glucose transporters 1 and 3. HIF1a-dependent upregulation of glycolysis facilitates rapid ATP generation under hypoxic conditions and produces intermediates for macromolecule synthesis, thereby promoting endothelial cell proliferation.[108]

Under hypoxia, HIF1α-induced upregulation of KLF4 promotes migration of vascular smooth muscle cells. As a result, the size of atherosclerotic lesions increases.[200] Irrespective of tissue oxygenation, HIF1a upregulation also increases the expression of osteopontin and monocyte

chemoattractant protein 1. Interestingly, however, HIF1α deficiency does not seem to affect LDL–induced foam cell formation or cholesterol efflux neither under normoxic nor under hypoxic conditions.[201]

Last but not least, HIF1a promotes the phenotypic switch of macrophages and monocytes towards the M1 pro-inflammatory phenotype. Interestingly, recent research conveyed that artesunate, a sesquiterpene lactone endoperoxide isolated from Chinese herbal medicine, displays anti-inflammatory activity by inhibiting HIF1a-mediated M1 macrophage polarization.[202]

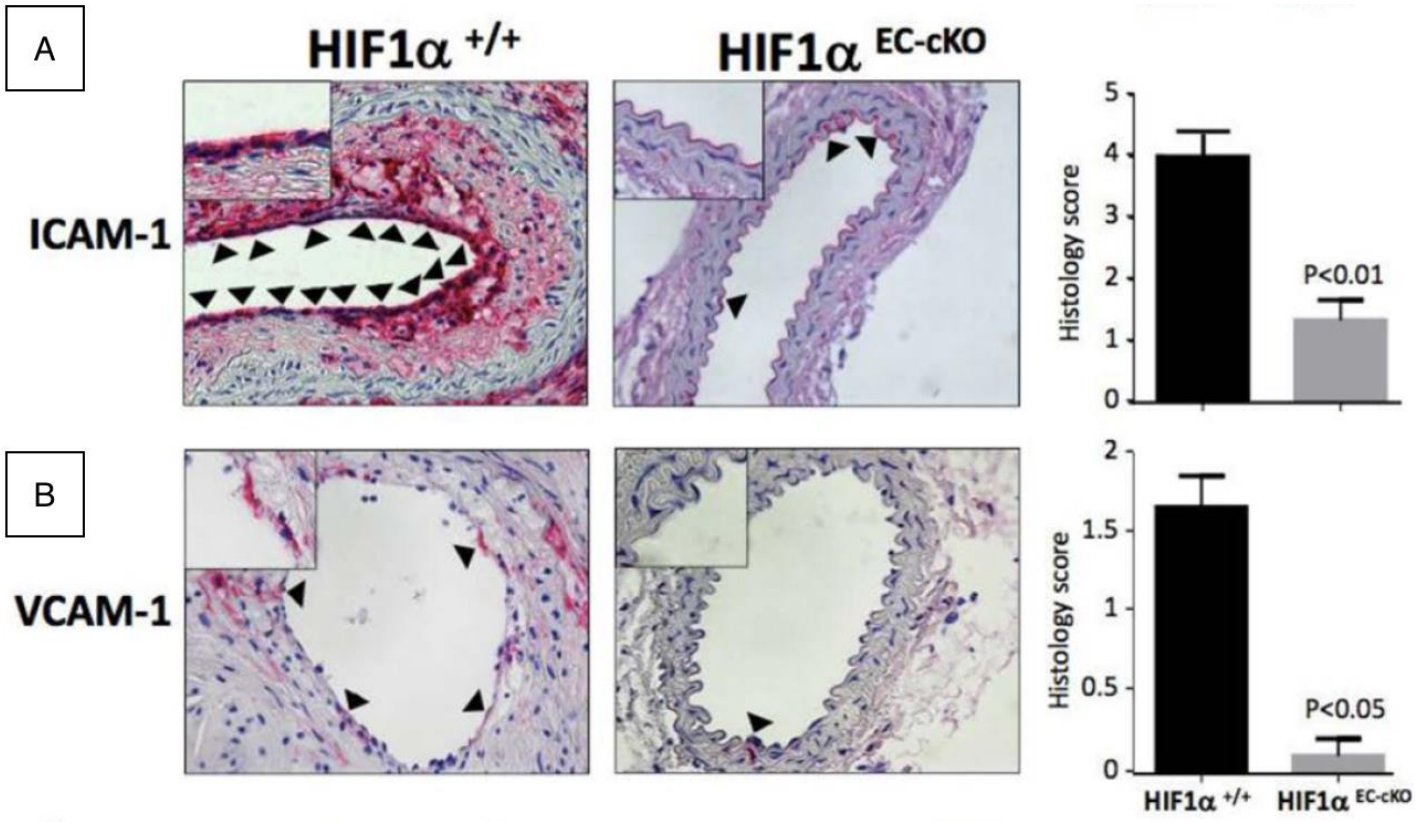


Figure G13.2 Increased expression of adhesion molecules ICAM1 and VCAM1 in response to HIF1a upregulation *(adapted from Feng S., et al., Mechanical Activation of Hypoxia-Inducible Factor 1a Drives Endothelial Dysfunction at Atheroprone Sites. Arterioscler Thromb Vasc Biol, 2017. 37(11): p. 2087-2*

CHAPTER 14

HOXA5

14.1 HOXA5 structure and function

Homeobox genes encode a class of transcription factors that regulate embryogenesis in multicellular organisms. These genes have been classified into four categories (A, B, C, and D) which are found on four separate chromosomes.[203] Homeobox gene expression is spatially and temporally regulated during embryonic development. *HOXA5* belongs to cluster A and is located on chromosome 7p15.2. *HOXA5* encodes a sequence-specific transcription factor which is part of a developmental regulatory system that dictates cellular identities on the anterior-posterior axis.[204] HOXA5 partly autoregulates its expression by binding to its own promoter. Particularly, it binds specifically to the motif 5'-CYYNATTA[TG]Y-3'.

HOXA5 is controlled, at least in part, by DNA methylation. Comparison of the *HOXA5* promoter methylation profile across cell types from the undifferentiated (human embryonic stem cells) to the most endothelial-like (e.g., human umbilical vein endothelial cells or HUVECs) shows that the *HOXA5* promoter is heavily methylated in non-differentiated cells and becomes demethylated as cells differentiate down the endothelial lineage. *HOXA5* contains a CREB element in its promoter.[8]

HOXA5 upregulates the tumor suppressor *p53* and *AKT1* genes by downregulating *PTEN*.[205] Suppression of *HOXA5* has been shown to promote tumorigenesis.[206] Furthermore, the overexpression of the HOXA5 protein prevents inflammation by inhibiting TNFα-induced monocyte binding.[207]

14.2 HOXA5 effects and role in cardiovascular disease

HOX family members are instrumental in maintaining vessel wall integrity, transmembrane signaling, cell cycle control, and vascular remodeling upon disruption.[208] HOX genes remain active postnatally and are centrally involved in angiogenesis. They work in regulatory networks and have a wide spectrum of interactions with various factors ranging from FGFs to integrins. Importantly, HOX expression is context-dependent and varies in different parts of the vascular bed.

HOXA5 is known to regulate specific endothelial functions including migration, angiogenesis, and inflammation by controlling specific genes (i.e., thrombospondin-2, vascular endothelial growth factor receptor-2, ephrin-A1, HIF1a, prostaglandin-endoperoxide synthase-2, and PTEN). *HOX5* upregulation promotes AJ stabilization by increasing the retention of beta-catenin in adherens junctions.[205]

HOXA5 is a mechanosensitive gene. Its expression is downregulated in a DNA methyltransferase 1 (DNMT1)–dependent manner in response to local endothelial inflammation, turbulent flow and low shear stress.[100] Downregulation of *HOXA5* expression has been implicated in vascular remodeling and derailed angiogenesis. It can also promote proatherogenic gene expression, extracellular matrix modification, and integrin alterations.

In the setting of *HOXA5* deficiency, macrophage cells switch towards the proinflammatory M1 phenotype. Vascular SMCs also undergo switching towards macrophage-type phenotypes.[209] Ultimately, reduced HOXA5 levels favor local vascular inflammation, intimal hyperplasia, and lipid accumulation. [210]

CHAPTER 15

α-Klotho

15.1 Klotho history

In 1997, Kuro-o et al. published their seminal study on a previously unknown gene, the mutation of which led to a clinical syndrome that resembled aging and entailed growth retardation, short lifespan, arteriosclerosis, ectopic calcification, osteoporosis, skin atrophy and emphysema in mice homozygous for the transgene. This gene was named *Klotho* after one of the three Fates in ancient Greek mythology (the Greek goddess who spins the thread of life). [211] Klotho levels appear to decline with aging and progressive chronic renal disease.[212, 213]

15.2 Klotho structure and function

Klotho is an anti-aging factor which participates in various biological processes from embryogenesis to adulthood. In humans, Klotho is mainly expressed in the distal tubule of the kidneys, parathyroid glands and the choroid plexus. Nevertheless, it has been recently demonstrated that human vascular tissue also expresses this protein.[212, 213] The *Klotho* gene is located on chromosome 13q13.1 and encodes three subfamilies of enzymes: α-Klotho (the most

abundant), β-Klotho, and γ-Klotho. The *Klotho* gene encodes a type-I membrane β-glucuronidase (130kDa) that is capable of hydrolyzing steroid β-glucuronides. The intermembrane form of α-Klotho acts a coreceptor for FGF23 when binding to its cellular receptor.[212]

A soluble form (65-70kDa) of Klotho is produced by cleavage of the membrane isoform by membrane metalloproteinases, such as A disintegrin and Metalloproteinase (ADAM)-17, or by direct secretion to the extracellular space of a shorter form derived from an alternative spliced transcript.[211] This soluble form has been shown to have hormonal properties independently from FGF-23. It is detectable in urine, serum and cerebrospinal fluid. It predominates in humans over the membrane form and acts as a humoral factor with multiple functions such as anti-oxidation, modulation of ion channels, anti-Wnt signaling or anti-apoptosis and anti-senescence effects.[214]

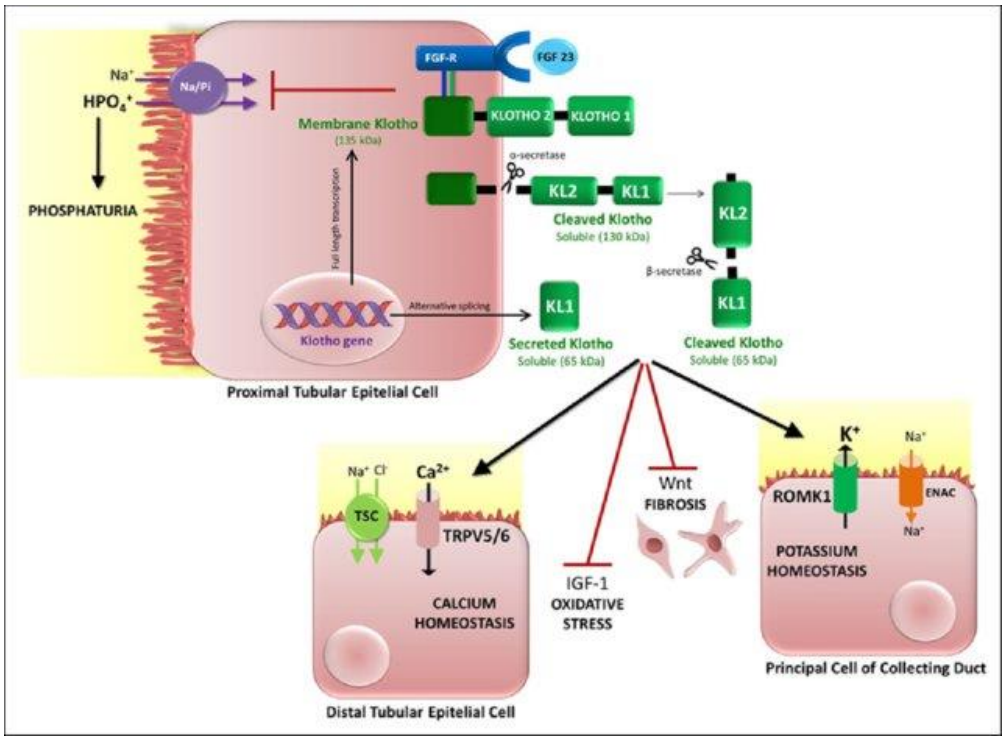


Figure G15.1 The effect of Klotho on calcium and phosphate homeostasis *(adapted from Andrade L. et al. Cell Transplantation. April 2018. 27(2):096368971774351)*

α-Klotho is a crucial component of the FGF-23/1,25-dihydroxyvitamin D/parathormone axis and plays a central role in regulating calcium and phosphate homeostasis (**Figure G15.1**).[215] More specifically, α-klotho changes cellular calcium homeostasis, by both increasing the expression and activity of TRPV5 (decreasing phosphate reabsorption in the kidney) and decreasing that of TRPC6 (decreasing phosphate absorption from the intestine). Klotho also stimulates calcium reabsorption in the distal tubule by preventing endocytosis, as well as by stabilizing the major calcium channels and the subfamily V (TRPV5 and TRPV6).[216, 217] Thus, Klotho works with FGF-23 to increase urinary phosphorus content, but it also ensures that urine with high phosphorus does not also have high calcium (thus preventing supersaturation of the urine). Both FGF-23 and Klotho are stimulated by 1,25-dihydroxyvitamin D (calcitriol), and both FGF-23 and Klotho inhibit renal 1α-hydroxylase (CYP27B1), thereby decreasing the conversion of 25-hydroxyvitamin D to calcitriol to complete the endocrine feedback loop.

15.3 The role of α-Klotho in atherogenesis

In cultured human umbilical endothelial cells, incubation with Klotho suppresses TNF α–induced ICAM1 and VCAM1 expression to halt monocyte adhesion. It also attenuates NF-κB activation.[218] Furthermore, the intracellular form of the Klotho protein can inhibit the retinoid acid-inducible gene (RIG)-I-induced expression of IL-6 and IL-8 both in vitro and in vivo.[219]

Klotho mediates inflammation of both endothelial cells and vascular SMC. Transfection of cultured vascular SMCs with Klotho has also been shown to reduce Nox2 NADPH oxidase protein expression whilst attenuating angiotensin II-induced superoxide production, oxidative damage,

and apoptosis.[220] Similarly, Klotho induces the expression of the antioxidant nuclear factor erythroid 2-related factor 2 (Nrf2) and the antioxidant enzymes heme oxygenase and peroxiredoxin-1. These enzymes ultimately enhance glutathione levels in human aortic SMCs.[221] On the contrary, pro-inflammatory cytokines like TNF-α and TNF-like weak inducer of apoptosis (TWEAK) can induce Klotho downregulation.[222]

A growing body of literature has also shown that α-Klotho protects against vascular calcification by preventing differentiation of vascular SMCs to an osteoblast-like phenotype.[223] Reversely, in Klotho-deficient cultured vascular SMCs, there was downregulation of the smooth muscle cell marker sm-22 and upregulation of the osteoblast marker Runx2 and sodium phosphate co-transporters Pit1 and Pit2. Therefore, Klotho deficiency seems to precondition cells to transform into osteoblast-like cells that are capable of initiating mineralization in response to phosphate uptake. Interestingly, vitamin D receptor activation by calcitriol or paricalcitol seems to restore Klotho and FGF-23 signaling and inhibit vascular calcification.[224, 225]

In adults without known risk factors for CVD, low serum Klotho has been associated with a reduced capacity of flow-mediated dilation of the brachial artery as well as higher values of epicardial fat thickness and carotid artery intima-media thickness, suggesting that reduced serum Klotho may be an early predictor of subclinical atherosclerosis.[226] Additional data suggest that patients with significant coronary disease not only present with lower serum Klotho concentrations, but also with reduced expression levels of Klotho mRNA in the coronary vessel wall (**Figure G15.2**).

The reduction in Klotho expression appears to be associated with the presence and severity of CAD independently of established cardiovascular risk factors.[227] In a multi-institutional study from Europe and the USA, myocardial biopsies from patients with a high 10-year

atherosclerotic CVD risk showed a significant reduction of Klotho expression as compared with cardiomyocytes from patients with a low ASCVD risk.[228]

Last but not least, as compared with control subjects, serum concentrations and vascular expression of Klotho were lower in patients with atherosclerotic vascular disease (i.e, aortic, carotid, peripheral), whereas inflammatory status was significantly higher. In addition, there was a negative and significant correlation between inflammatory parameters and Klotho. After controlling for the effect of other variables, direct relationship between vascular Klotho gene expression and IL-10 mRNA levels was identified. On the other hand, there was a negative association between serum LDL concentrations and vascular TNF-α expression.[229]

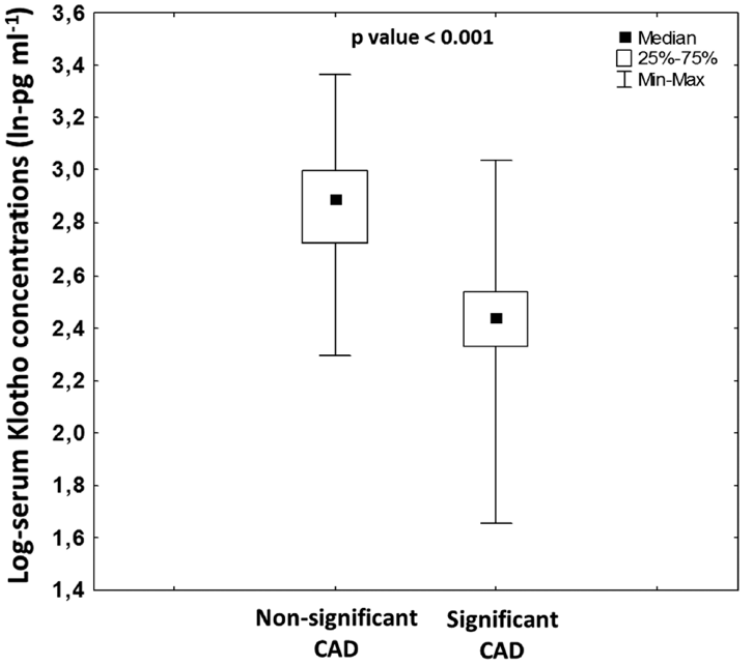


Figure G15.2 Differences in the log-transformed levels of serum Klotho concentrations in patients with significant vs non-significant coronary artery disease *(adapted from Navarro-González J.F. et al., Heart, 2014. 100(1): p. 34-40.)*

CHAPTER 16

The rabbit as an experimental model of atheromatosis

Rabbits are frequently utilized as “large animal” models in experimental atherosclerosis research.[230-232] A wide spectrum of dietary treatments have been employed to investigate the effects of hypolipidemic and anti-inflammatory medication on atherosclerotic processes.[230-232]

A growing body of literature suggests that the thoracic and abdominal aortas of rabbits develop fatty streaks and atheromata relatively soon after the administration of hypercholesterolemic diet.[233] Indeed, it is relatively simple to induce atherosclerotic lesions in these animals by feeding them a high-cholesterol diet. Time-to-onset of measurable atheromatosis ranges from 4 to 16 weeks depending on the amount of cholesterol added to the usual chow.[234] Particularly the White New Zealand rabbit has been found to develop significant atheromatosis following diet enriched with 0.5%-2% cholesterol for a period of six to eight weeks. [235] Other reports have shown that atherogenesis may be triggered by enriching feeds with as low as 0.1% cholesterol.[236]

Rabbits develop atheromatosis rapidly because they are unable to effectively expel sterols. The accumulation of cholesterol induces the hepatic production of lipoproteins which ultimately accumulate in atheroprone regions. To accelerate the process of atherogenesis, certain groups have combined cholesterol-enriched diets with mechanical intimal injury (i.e., by traumatizing the

endothelium with an intravascular balloon).[237] Furthermore, certain rabbit breeds (e.g., St. Thomas, Watanabe, and Houston RT) have been genetically modified to bear defects in various steps of lipid metabolism making them susceptible to atheromatosis.[238]

Lipoprotein processing in rabbits resembles human physiology in many ways, including apolipoprotein B synthesis, apo B_{100} production, and cholesteryl-ester transfer protein activity.[233, 236] Watanabe rabbits are characterized by defective LDL receptors and have been utilized as experimental models to study familial hypercholesterolemia.[238] That being said, lipid metabolism in rabbits also has certain differences compared to humans. These include lack of hepatic lipase and the risk of hepatotoxicity in the setting of prolonged exposure to cholesterol-enriched diet.[232, 239]

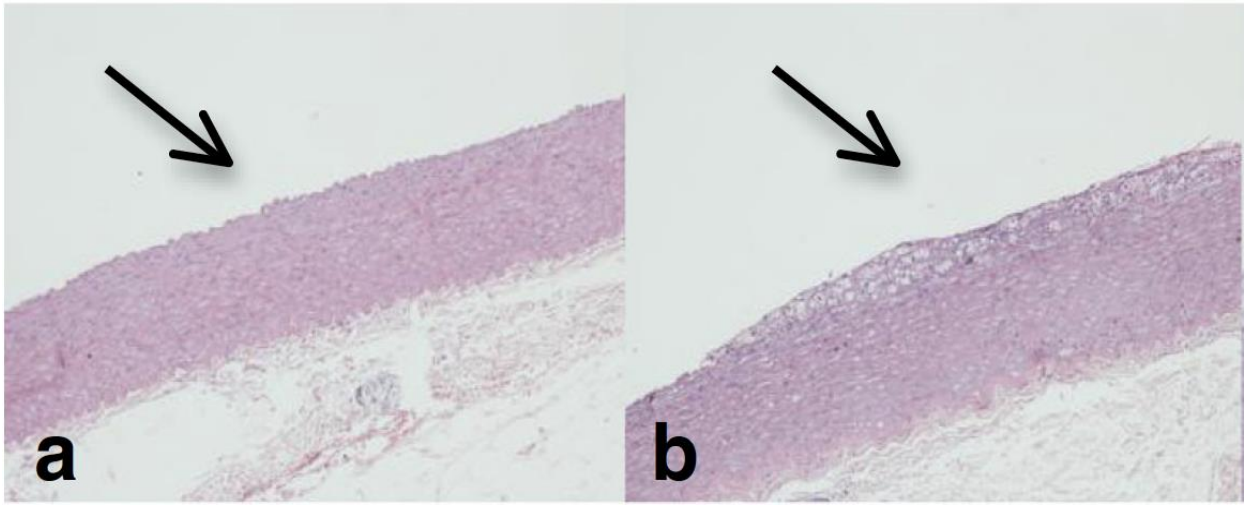


Figure G15.2 Hematoxylin-eosin image of thoracic aorta specimens indicating the presence of preliminary atheromatosis in rabbits fed with 1% w/w cholesterol-enriched diet (b) compared to controls (a) *(adapted from Kaminiotis V.V., et al., Lipids Health Dis, 2017. 16(1): p. 184.*

Experiment

CHAPTER 17

Materials and methods

17.1 Purpose

White New Zealand rabbits were utilized to develop an animal model of atheromatosis. The purpose of the experiment was to assess the impact of anti-inflammatory treatment on curtailing the *de novo* development of thoracic and abdominal aortic atheromatosis during a predefined period of prolonged hyperlipidemia. The primary endpoints of the study were to explore the effect of colchicine-based regimens on:

a) pro-atherogenic inflammation and the development of atheromatosis
b) as well was the potential impact of anti-inflammatory therapy on the expression patterns of the following mechanosensitive stem cell genes:

1. KLF4
2. BMP4
3. SOX2
4. OCT4
5. NANOG
6. NOTCH1
7. HIF1A
8. HOXA5
9. Klotho-a

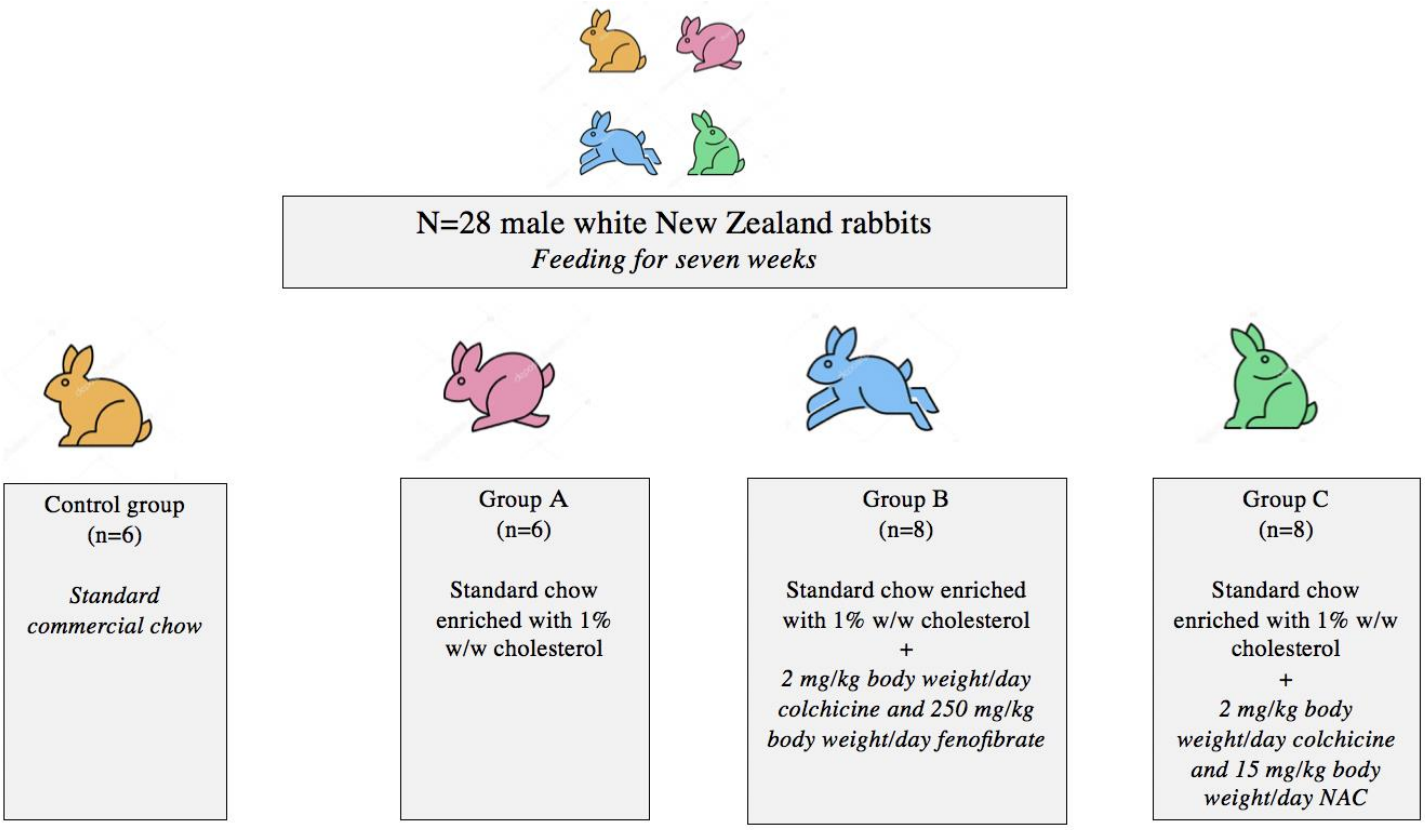
17.2 Animal model and experimental design

Twenty-eight male, eight-week-old, New Zealand White rabbits (*Oryctolagus cuniculus*) (3.65 ± 0.23 kg) were obtained. The animals were donated by an Attica-based farm that breeds rabbits for experimental reasons. The study animals were housed one per cage in an air-conditioned facility (19 ± 2 °C with 60 ± 5% relative humidity and 15 air changes/hour). A 12-hour light/dark cycle (5:30 am to 5:30 pm) was enforced. All animals had unobstructed access to food and tap water. Daily consumption of tap water was measured individually per rabbit. Any intervention that could potentially cause discomfort to the animals was performed under mild ketamine-xylazine sedation. All study subjects were treated in accordance with current European Council Directives (276/33/20.10.2010). The study protocol was approved by the Veterinary Directorate of the Prefecture of Athens (Approval No.: 3231/26.06.2018). Institutional Board Review (IRB) approval was also obtained by the Ethics Committee of the School of Medicine of the National and Kapodistrian University of Athens.

Following a two-week acclimatization period, study subjects were divided in four experimental groups (**Figure M17.1**). Animals in the control group (n = 6) were fed standard commercial rabbit chow [Conigli Svezzamento, S.I.V.A.M. Società Italiana Veterinaria Agricola Milano S.P.A., Casalpusterlengo (LO), Italy] which consisted of 37% carbohydrates, 16% protein, 4% fat, 15% fiber, 11% water, and 8% ash. Animals in group A (n = 6) were fed standard diet enriched with 1% w/w cholesterol. The hyperlipidemic food was prepared by dissolving the appropriate amount of cholesterol in diethyl ether (without butylated hydroxytoluene as inhibitor) and the mixture was subsequently added to the rabbit chow. After ether evaporation, the cholesterol-enriched food was kept at −20 °C until use.

Group B animals (n=8) were fed the same cholesterol-enriched diet plus 2 mg/kg body weight/day colchicine and 250 mg/kg body weight/day fenofibrate. Lastly, Group C animals (n=8) were fed the same cholesterol-enriched diet plus 2 mg/kg body weight/day colchicine and 15 mg/kg body weight/day N-acetylcysteine (NAC). Colchicine, fenofibrate, and NAC were pulverized and then dissolved in tap water. All three medications in the aforementioned doses have been shown to affect atherosclerotic plaques in hyperlipidemic rabbit models.[80, 234, 235, 240, 241].

Figure M17.1 Experimental study groups



Of note, the effects of colchicine vs placebo on atherosclerotic plaque stabilization in hyperlipidemic rabbit models were recently reported.[242] On Positron Emission

Tomography/Computed Tomography (PET/CT), maximum 18F-Fluorodeoxyglucose (18F-FDG) standardized uptake value decreased after colchicine treatment. On Magnetic Resonance Imaging (MRI), plaque volume burden was assessed as the sum of the vessel wall area of each slice (outer wall area minus lumen area. Results were expressed as normalized wall index (NWI), derived as aortic wall volume divided by the total vessel volume.

Colchicine led to a significant reduction in NWI. Histological analyses revealed that colchicine may stabilize established atherosclerotic plaque by reducing inflammatory activity and plaque burden, without altering macrophage infiltration or plaque typology.

17.3 Tissue preparation

No unplanned fatality occurred during the feeding period in any of the experimental groups. At the seven-week time mark, all study animals were sedated with intramuscular ketamine-xylazine and subsequently euthanized via intravenous administration of sodium pentobarbital (120mg/kg). [230] Aortas were surgically excised from the arch down to the iliac bifurcation. (**Figure M17.2**).

Perivascular adipo-connective tissue was dissected off of the vessels. Thoracic aortas were sampled between the origin of the brachiocephalic vessels and the $3^{rd}/4^{th}$ intercostal arteries. Abdominal aortas were sampled between the diaphragm and the iliac bifurcation. Tissue specimens were maintained in a 10% neutral buffered formalin solution for 24 hours and were afterwards embedded in paraffin blocks maintaining native vertical orientation.

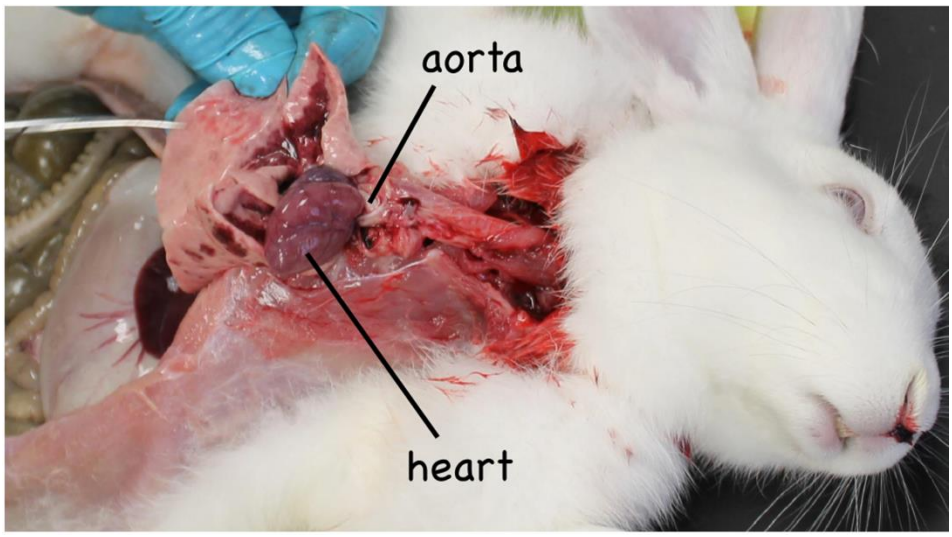


Figure M17.2 Gross aortic phenotype in the setting of no atheromatosis (image representative of the control group)

17.4 Histology

Ten serial paraffin slices of 5 μm thickness were cut along the thoracic and abdominal aortic samples and stained with eosin and hematoxylin (H&E). The histologic examination was done by an expert who was blind to the intervention groups.

17.5 Morphometric Analysis

The specimens were examined under a Leica DMLS2 light microscope (Leica Microsystems Wetzlar GmbH, Germany). Digital images were acquired using a Leica DFC500 digital color camera (working resolution: 4,080 × 3,072 pixels) and the Leica LAS 3.6 software. A morphometric analysis of thoracic and abdominal aortic segments was performed for the

quantification of atherosclerotic plaques in ImagePro Plus version 5.0 (Media Cybernetics, Bethesda, MD, USA).

A previously validated institutional protocol was used to perform the morphometric analysis.[243] Atherosclerotic plaque area (μm^2) was measured in the H&E-stained sections by obtaining morphometric measurements of the surface area of the lesion and the length of the internal elastic lamina (IEL).[244] Measurements of the adventitia, tunica media, fibrous cap, and lesion shoulders were excluded from the analysis.[243]

The extent of the atherosclerotic plaques (μm^2) was measured. Total lumen area (μm^2) was calculated by measuring the IEL perimeter and extrapolating the circumscribed area for each section. Lumen area could not be measured directly using images of non-perfectly circular aortic cross-sections. Nevertheless, it was assessed indirectly, by measuring the internal perimeter of the lumen which remains unchanged regardless of tissue folding.

Spherical lumen area was subsequently derived using the following formula: $A=P^2/4\pi$, where A=the lumen area and P=the measured perimeter, π=the mathematical constant of the ratio of a circle's circumference to its diameter. Again, the IEL distinguished plaque from arterial wall. The proportion of calculated total lumen area occupied by the atherosclerotic plaques expressed the percentage of luminal stenosis, in each section. For atherosclerotic lesion quantification, we averaged plaque and lumen area and the luminal stenosis from all hematoxylin/eosin-stained sections per animal.

17.6 Biochemical measurements

At 0 (T0), four (T1) and seven (T2) weeks of dietary intervention, blood samples were drawn from the central ear artery following overnight fasting. These samples were collected for the determination of total cholesterol (TC), HDL cholesterol, LDL cholesterol, triglycerides, glucose, creatinine, total protein, γ-GT, alkaline phosphatase (ALP), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels. Samples were stored at −80 °C until analysis.

17.7 RNA extraction from vascular tissue-cDNA synthesis

Thoracic and abdominal aortic tissue samples were placed in RNAlater® solution (Ambion (Europe) Limited, UK) and stored at 4°C for subsequent RNA extraction. In total, 100-200 mg of tissue was utilized from each experimental animal (50-100 mg thoracic aorta and 50-100 mg abdominal aorta).

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, CA, USA) according to the manufacturer's instructions. [229] All samples were homogenized and then centrifuged for 10 minutes at 12,000xg at 4^{o}C. Samples were subsequently incubated for 5 minutes at room temperature to achieve complete separation of the nucleoprotein complex.

Then, 200 μl of chloroform were added per 1 ml of trisol used for homogenization. After additional incubation for 2-3 minutes at room temperature, a second centrifugation was performed at 13,000rpm for 15 minutes at 4°C. The aqueous phase was transferred to new tubes. RNaseZap® was used to prevent RNA contamination. 500 μl of 100% isopropanolol was added to isolated samples per 1 ml of trisol and the samples were incubated for 16 hours.

Moving forward, the samples were placed in the vortex and centrifuged for 10 minutes at 12,000xg at 4^{o}C. Supernatants were removed and only the RNA pellet was retained. This was

washed with 1 ml of 75% ethyl alcohol for every 1 ml of trisol used for homogenization. Study samples were repositioned in the vortex and then centrifuged for 5 minutes at 7,600xg at 4°C. The resulting supernatants were discarded, and the RNA pellet was redissolved with 30 µl of free water.

RNA was quantified using a Thermo Scientific NanoDrop™ Lite Spectrophotometer (Thermo Scientific, MA, USA) and its quality was assessed by the A_{260}/A_{280} ratio measured in this equipment. Complementary DNA (cDNA) was produced when the A_{260}/A_{280} ratio was greater than 1.8. [229]

17.8 Quantitative real-time PCR (qRT-PCR)

Quantitative real-time PCR was utilized.[229] cDNA was synthesized using the PrimeScript RT reagent kit (Takara Bio, Japan) according to the manufacturer's protocol.

The qPCR reaction was performed using cDNA along with KAPA SYBR® FAST qPCR Master Mix (2X) Kit (KK4602, Sigma-Aldrich), forward/reverse primers and water. To ensure the validity of the results, each sample was analyzed three times (triplicates) using three separate wells of 20µl each. All genes of interest for all experimental animals were analyzed at the same time along with a control gene that is universally expressed in cells (glyceraldehyde 3-phosphate dehydrogenase - GAPDH) (housekeeping gene).

Amplification of cDNA (C_T) values were estimated for all reactions. The level of target mRNA was estimated by relative quantification using the comparative method ($2^{-\Delta\Delta Ct}$) by normalizing to GAPDH expression. The following primer sequences were utilized:

KLF4

Sequence (5'->3')	Sequence (5'->3')	Template strand
Forward primer	GGACCTACGTACTCGCCTTG	Plus
Reverse primer	CAGTGAGGGTACCCGAAACC	Minus
Product length	151	

BMP4

	Sequence (5'->3')	Template strand
Forward primer	GGGCTTCCACCGGATAAACA	Plus
Reverse primer	CCTGATGGGTCCGTGTATGG	Minus
Product length	227	

SOX2

	Sequence (5'->3')	Template strand
Forward primer	CTACAGCATGATGCAGGACCA	Plus
Reverse primer	TGGGAGAGCCGTTCATGTAG	Minus
Product length	155	

OCT4

	Sequence (5'->3')	Template strand

Forward primer	GCTCCCCCATGCATTCAAAC	Plus
Reverse primer	ACTGAATCCCCAAAGCCCTG	Minus
Product length	117	

NANOG

	Sequence (5'->3')	**Template strand**
Forward primer	TACCCAGGCTTCTACCCTGC	Plus
Reverse primer	GGTTACTCCACGACTGGCTG	Minus
Product length	136	

NOTCH1

	Sequence (5'->3')	**Template strand**
Forward primer	GTTCGAGGACCAGATGGCTT	Plus
Reverse primer	GATGTTGGCATCTGCACTGG	Minus
Product length	243	

HIF1α

	Sequence (5'->3')	**Template strand**
Forward primer	TTTTGGCAGCAACGACACAG	Plus
Reverse primer	GTGCAGGGTCAGCACTACTT	Minus
Product length	173	

HOXA5

	Sequence (5'->3')	**Template strand**
Forward primer	CATGCTCTTTGCCTCTCCGA	Plus
Reverse primer	TTAGGGCAACGAGAACAGGG	Minus
Product length	248	

a-Klotho

	Sequence (5'->3')	**Template strand**
Forward primer	CAGCGACGGCTACAACAATG	Plus
Reverse primer	AGCAGAGTTCGGCGTAATCC	Minus
Product length	296	

GAPDH

	Sequence (5'->3')	**Template strand**
Forward primer	AGGGCTGCTTTTAACTCTGGT	Plus
Reverse primer	CCCCACTTGATTTTGGAGGGA	Minus

17.9 Statistical analysis

Continuous variables were summarized using means ± standard deviations (SD). Data distributions were assessed with the Shapiro-Wilk test. Due to the limited sample size and non-normality of the distributions of somatometric and biochemical data, non-parametric tests were utilized. Specifically, the Wilcoxon paired-sample test and Mann-Whitney U test were performed

to statistical analyze the aforementioned variables. Benjamini Hochberg's false discovery rate was used to determine pairwise significance levels while accounting for family-wise type I errors in all situations involving multiple comparisons of somatometric or biochemical data.

For PCR data, one-way analysis of variance (ANOVA) was performed with Tukey's correction to account for multiple comparisons. Mean differences with 95% confidence intervals (95% CI) were calculated. A p-value <0.05 was considered statistically significant. All p-values were two-sided. All statistical calculations were performed using Stata/BE 17.0 for Mac (StataCorp, 4905 Lakeway Drive, College Station, TX, USA) and GraphPad Prism version 4.03 (GraphPad Inc, CA, USA).

CHAPTER 18

Results

18.1 Somatometric effects

Rabbit weights at the fourth (T1) and seventh (T2) week of the experiment were significantly higher compared to baseline in all study groups. Nevertheless, no statistically significant differences were noted at T1 and T2 in terms of body weight. Similarly, neither the administration of hyperlipidemic diet nor colchicine-based interventions influenced body weight gains in any of the study arms (**Table R18.1**).

18.2 Lipidemic measurements

Lipidemic measurements were comparable at the beginning of the experiment among all study groups (**Table R18.1**). Both at T1 and at T2, total cholesterol and LDL levels were significantly higher in all three hyperlipidemic groups compared to the control arm. By the end of the experiment, HDL values were significantly higher in animals that underwent colchicine-based interventions compared to baseline and T1. In addition, at seven weeks, the colchicine/fenofibrate and colchicine/NAC arms had significantly lower triglyceride levels compared to baseline and to

the cholesterol group. The addition of fenofibrate to colchicine produced a more robust reduction in triglycerides compared to NAC. At the end of the experiment, there was a statistically insignificant trend towards higher triglyceride levels in the cholesterol diet group A **(Table R18.1)**.

Table R18.1. Somatometric and lipidemic measurements

	Group	***Baseline***	***4 weeks***	***7 weeks***
Body weight (kg)	Control	3.6 ± 0.1	4.3 ± 0.3[a]	*4.2 ± 0.2[a,b]*
	Cholesterol	3.6 ± 0.2	4.3 ± 0.3[a]	*4.3 ± 0.3[a,b]*
	Col + Fen	3.5 ± 0.1	4.2 ± 0.2[a]	*4.0 ± 0.2[a,b]*
	Col + NAC	3.6 ± 0.1	4.1 ± 0.2[a]	*4.0 ± 0.2[a,b]*
Total cholesterol (mg/dL)	Control	45.8 ± 10.7	44 ± 6.7	*40.7 ± 9.8*
	Cholesterol	58.2 ± 19.7	2163.3 ± 675.1[a,c]	*2096.7 ± 663.4[a,b,c]*
	Col + Fen	40.6 ± 10.2	2496.3 ± 745[a,c]	*2460 ± 728.1[a,b,c]*
	Col + NAC	44.8 ± 10.2	3176.3 ± 515.6[a,c]	*3112.5 ± 512.5[a,b,c]*
HDL (mg/dL)	Control	34.3 ± 10.4	33.8 ± 10.14	*33.3 ± 9.7*
	Cholesterol	36.3 ± 11.1	141.7 ± 45[a,c]	*139.8 ± 32.5*
	Col + Fen	33.9 ± 10.2	143.8 ± 26.1[a,c]	*143.3 ± 21.6[a,b,c]*
	Col + NAC	28.3 ± 4.8	136.3 ± 20.7[a,c]	*132.3 ± 20.6[a,b,c]*
LDL (mg/dL)	Control	20.2 ± 2.9	20.3 ± 2.4	*1.3 ± 1.1*
	Cholesterol	10.5 ± 10.1	1998.8 ± 653.7	*1956.3 ± 212.1*
	Col + Fen	26.3 ± 2.6	2337.1 ± 743.1	*2356.3 ± 212.1[a,b,c]*
	Col + NAC	50.2 ± 12.1	3034.5 ± 493.3	*2856.3 ± 212.1[a,b,c]*
Triglycerides (mg/dL)	Control	96.3 ± 14.9	96.7 ± 11.3	*94.8 ± 14.3*
	Cholesterol	90.8 ± 23.3	114.2 ± 30.7	*119 ± 24.4*
	Col + Fen	97.2 ± 8.4	56.9 ± 18.9	*49.5 ± 14.6[a,b,c,d,e]*
	Col + NAC	97.5 ± 16.9	70 ± 14.9	*58.3 ± 12.3[a,b,c,d]*

Paired samples Wilcoxon test adjusted with Benjamini-Hochberg procedure.
†Data are presented as mean ± SD, [a]p < 0.05 vs. baseline, [b]p < 0.05 vs. 4 weeks, [c]p < 0.05 vs. Control group, [d]p < 0.05 vs. Cholesterol group, [e]p < 0.05 vs. vs. Col + NAC.

18.3 Liver and renal function measurements

During the course of the experiment, no significant differences were observed in terms of SGOT, SGPT, γGT, ALP, and creatinine in any of study groups **(Table R18.2)**.

Table R18.2. Liver and renal function measurements

	Group	*Baseline*	*4 weeks*	*7 weeks*
SGOT (U/L)	Control	19.2 ± 5.9	22.3 ± 4.8	*23.5 ± 6*
	Cholesterol	20.2 ± 8.3	22 ± 9.6	*20.3 ± 8.7*
	Col + Fen	14.3 ± 3.0	21.5 ± 7.5	*24.4 ± 7.6*
	Col + NAC	20.8 ± 5.8	26.4 ± 2.3	*26.1 ± 3.5*
SGPT (U/L)	Control	42.8 ± 12.1	42.8 ± 11.7	*43.2 ± 11.8*
	Cholesterol	46 ± 24	45.5 ± 20.4	*46.1 ± 18.7*
	Col + Fen	39.9 ± 18.7	42 ± 10.5	*42 ± 11.5*
	Col + NAC	42.4 ± 15.9	47.6 ± 14.5	*45 ± 13.2*
γGT (U/L)	Control	11.3 ± 1.2	10 ± 1.3	*9.3 ± 1.2*
	Cholesterol	11.3 ± 2.6	10.8 ± 3.4	*10.3 ± 3.*
	Col + Fen	11.1 ± 2.0	10.8 ± 1.4	*10.1 ± 1.4*
	Col + NAC	10.5 ± 1.4	10.1 ± 1.5	*9.5 ± 0.9*
ALP (U/L)	Control	141 ± 16.4	137.8 ± 19.1	*130.3 ± 21.3*
	Cholesterol	145.5 ± 29.2	144.8 ± 27.2	*140.7 ± 21.5*
	Col + Fen	153.1 ± 17.9	148.8 ± 18.5	*146.4 ± 18.1*
	Col + NAC	131.1 ± 25	137.9 ± 17.7	*136.8 ± 14.1*
Creatinine (mg/dL)	Control	1 ± 0.06	1.05 ± 0.08	*0.98 ± 0.07*
	Cholesterol	1.01 ± 0.14	1.24 ± 0.10	*1.18 ± 0.13*
	Col + Fen	0.91 ± 0.11	1.05 ± 0.05	*1.01 ± 0.08*
	Col + NAC	0.91 ± 0.08	1.05 ± 0.07	*1.02 ± 0.1*

18.4 Atheromatous plaque extent in the thoracic aorta

At the end of the experiment, animals in the control group did not exhibit atherosclerotic lesions in their thoracic aortas (**Figure R18.1**). Group A was fed atherogenic diet for seven weeks without any pharmacologic intervention which led to significantly more extensive atherosclerosis compared to both animals that received colchicine/fibrate (MD: 13.7, 95% CI: 7.5 to 19.8, $p<0.001$) and to those that were treated with colchicine/NAC (MD: 20.3, 95% CI: 14.1 to 26.5, $p<0.001$). The co-administration of colchicine with NAC resulted in significantly greater reduction

in the extent of thoracic aortic atherosclerotic plaques compared to the colchicine/fibrate regimen (MD: 6.6, 95% CI: 0.9 - 12.3), **(Table R18.3, Figures R18.1-R18.2**).

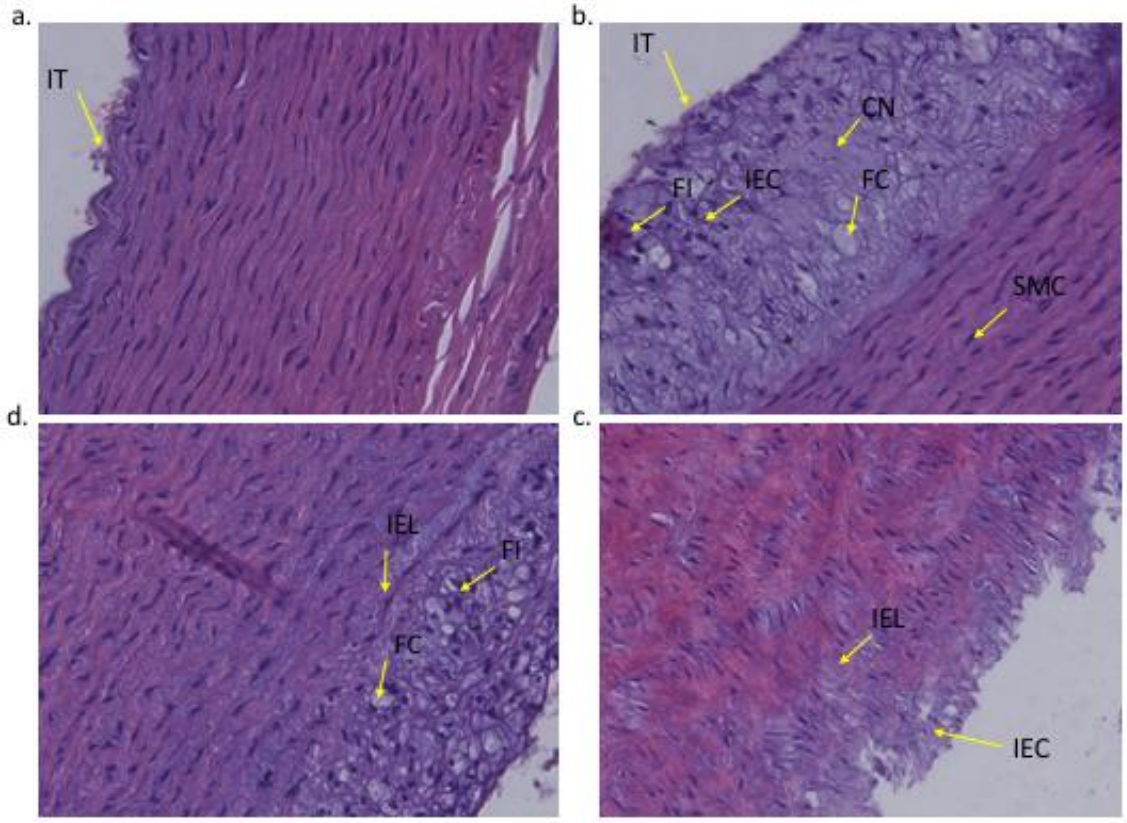


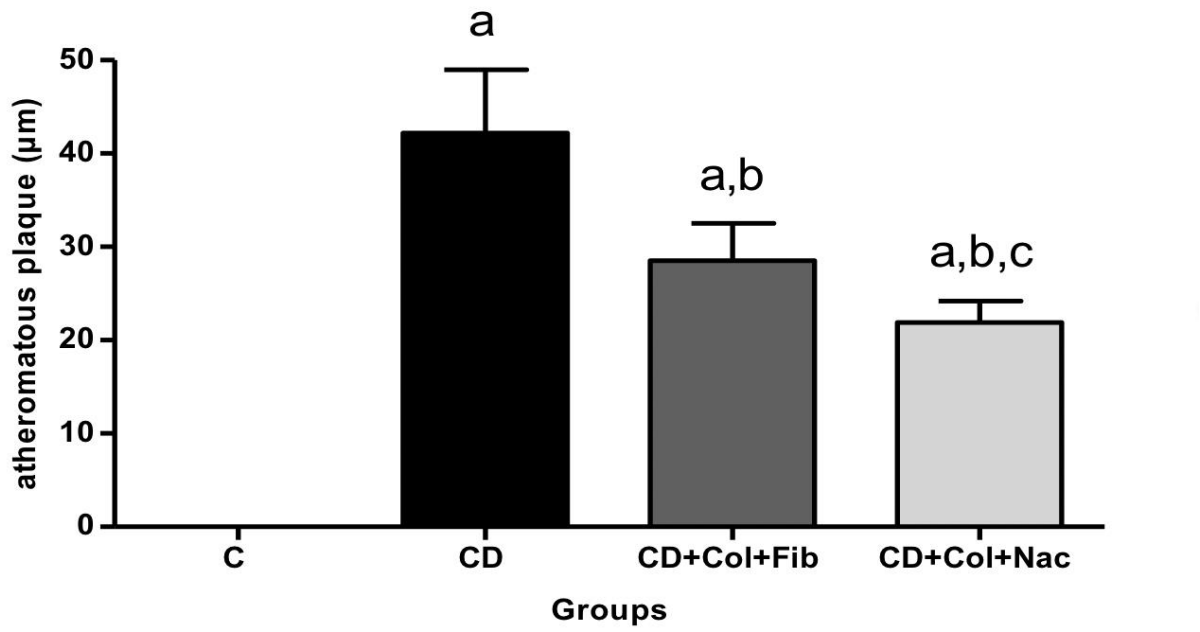
Figure R18.1 Histologic analysis of the rabbit thoracic aorta. Representative pictures of Hematoxylin and eosin staining. a. Control group: normal thoracic aorta, no atheromatous plaque; b. Cholesterol group: typical atherosclerotic plaque formation characterized by fibroatheroma, full of multiple lipid cores vacuolized in cytoplasm, accumulation of smooth muscle cells and fibrosis; c. group B: atheromatous plaque with small area of extracellular foam cells; d. group C: atheromatous plaque with accumulation of endothelial cells. M*agnification 40 ×. Abbreviations: internal tunica, (IT), injury to endothelial cells (IEC), cellular necrosis (CN), foam cells (FC), smooth muscle cells (SMC), infiltration of inflammatory cells (IC), proliferation of fibroblasts (FI), internal elastic lamina (IEL).*

Table R18.3. Morphometric analysis of atherosclerotic plaques in thoracic aortas

ANOVA with Bonferroni's correction	Mean Difference	95% CI of difference	p-value
C vs CD	-42.2	-48.8 to -35.6	<0.001
C vs CD+Col+Fib	-28.5	-34.7 to -22.3	<0.001
C vs CD+Col+NAC	-21.8	-28.1 to -15.7	<0.001
CD vs CD+Col+Fib	13.7	7.5 to 19.8	<0.001
CD vs CD+Col+NAC	20.3	14.1 to 26.5	<0.001
CD+Col+Fib vs CD+Col+NAC	6.6	0.9 to 12.3	<0.001

ANOVA: analysis of variance; 95% CI: 95% confidence interval; C: control, CD: cholesterol diet (group A), Col: colchicine, Fib: fibrate, NAC: N-acetylcysteine

Figure R18.2. Morphometric analysis of atherosclerotic plaques in rabbit thoracic aortas



C: control, CD: cholesterol diet (group A), Col: colchicine, Fib: fibrate, NAC: N-acetylcysteine, a: Control vs A, Control vs B, Control vs C, b: A vs B, A vs C, c: B vs C

18.5 Atheromatous plaque extent in the abdominal aorta

Similar findings were observed in terms of abdominal aortic atherogenesis. Indeed, at the end of the experiment, animals in the control group did not exhibit any atherosclerosis or fatty streaks in their abdominal aortas. Animals in the unmedicated hyperlipidemic Group A had significantly more extensive atherosclerosis compared to group B (MD: 12.6, 95% CI: 7.1 to 18.2, $p<0.001$) and group C (MD: 23.8, 95% CI: 18.2 to 29.3, $p<0.001$), **(Figure R18.3**). Again, the co-administration of colchicine with NAC resulted in significantly more robust reduction in the extent of abdominal aortic atheromatosis compared to the colchicine/fibrate regimen (MD: 11.1, 95% CI: 6.0 – 16.3), **(Table R18.4, Figures R18.3-R18.4)**.

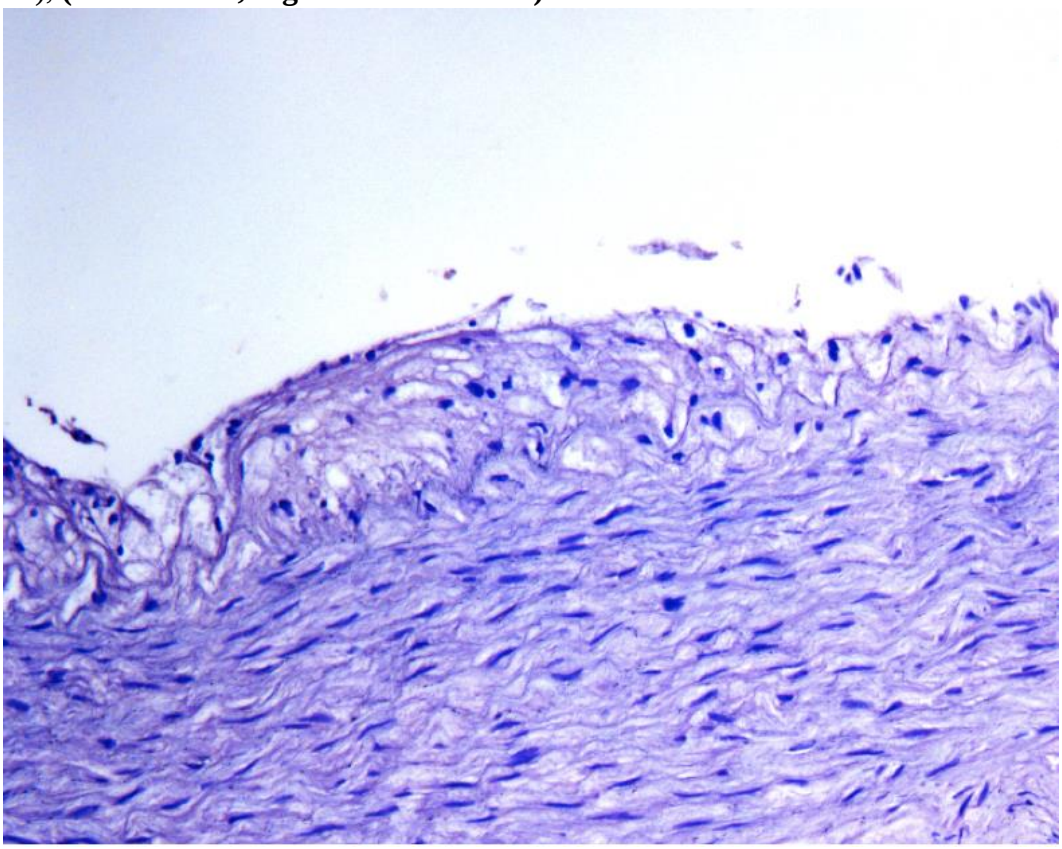


Figure R18.3 Histologic analysis of the rabbit abdominal aorta. Representative pictures of Hematoxylin and eosin staining. Cholesterol group: typical atherosclerotic plaque formation

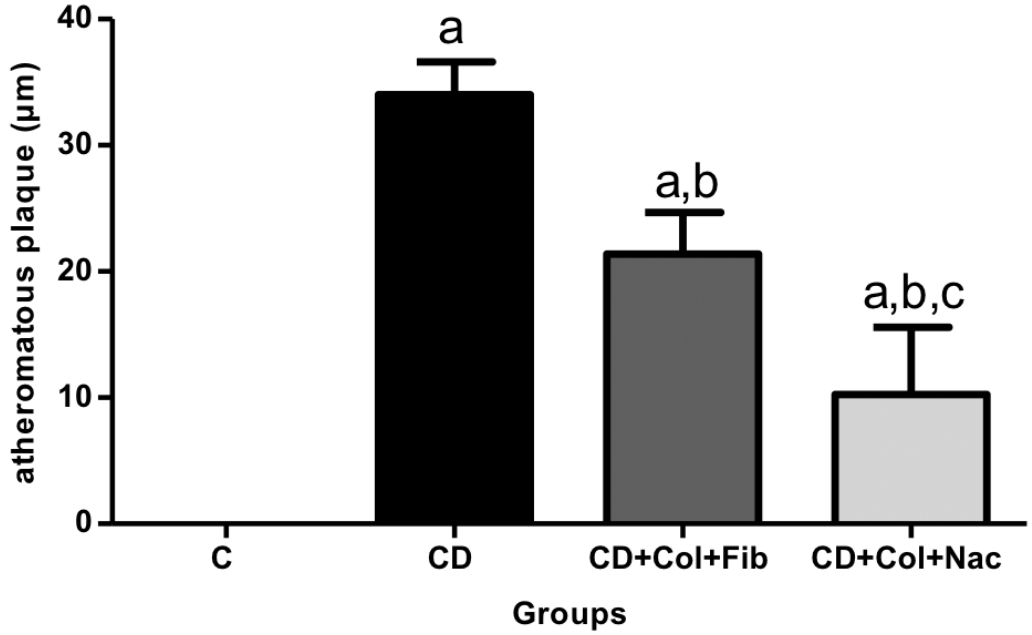
characterized by fibroatheroma, full of multiple lipid cores vacuolized in cytoplasm, accumulation of smooth muscle cells and fibrosis; M*agnification 20 ×*.

Table R18.4. Morphometric analysis of atherosclerotic plaques in abdominal aortas

ANOVA with Bonferroni's correction	Mean Difference	95% CI of difference	p-value
C vs CD	-34.0	-40.0 to -28.0	<0.001
C vs CD+Col+Fib	-21.4	-27.0 to -15.8	<0.001
C vs CD+Col+NAC	-10.3	-15.8 to -4.7	<0.001
CD vs CD+Col+Fib	12.6	7.1 to 18.2	<0.001
CD vs CD+Col+NAC	23.8	18.2 to 29.3	<0.001
CD+Col+Fib vs CD+Col+NAC	11.1	6.0 to 16.3	<0.001

ANOVA: analysis of variance; 95% CI: 95% confidence interval; C: control, CD: cholesterol diet (group A), Col: colchicine, Fib: fibrate, NAC: N-acetylcysteine

Figure R18.4. Morphometric analysis of atherosclerotic plaques in rabbit abdominal aortas



C: control, CD: cholesterol diet (group A), Col: colchicine, Fib: fibrate, NAC: N-acetylcysteine, a: Control vs A, Control vs B, Control vs C, b: A vs B, A vs C, c: B vs C

18.6 KLF4 expression

18.6.1 KLF4 in thoracic aortic specimens

Minimal *KLF4* expression was noted in the thoracic aortas of the control group (mean $2^{-\Delta\Delta ct}$=1.81, SD:0.47). The *KLF4* gene was overexpressed in all hyperlipidemic animals (**Table R18.5**). The unmedicated rabbits of group A (mean $2^{-\Delta\Delta CT}$ =25.88, SD:3.57) exhibited significantly greater *KLF4* expression compared to those in group B (MD: 4.94, 95% CI: 1.11 to 8.77) and group C (MD: 9.94, 95% CI: 6.11 to 13.77, p<0.001). Combining colchicine with NAC instead of fenofibrate (MD: 5.00, 95% CI: 1.45 to 8.54, p<0.001) led to a more robust reduction in thoracic aortic expression of *KLF4* **(Table R18.6.1, Figure R18.5**).

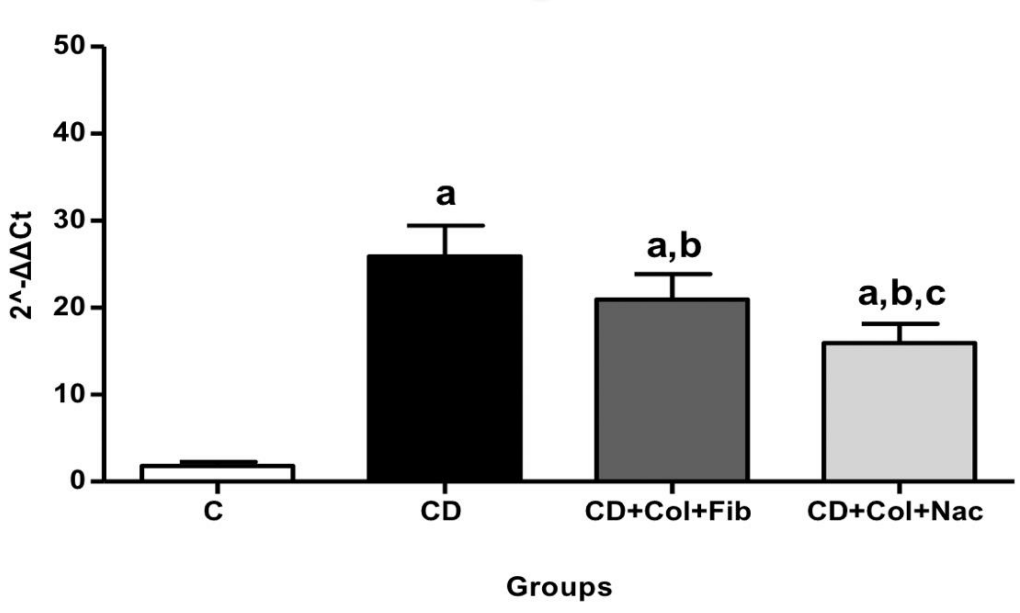
Table R18.5. *KLF4* expression levels ($2^{-\Delta\Delta CT}$) in rabbit thoracic aortas

Study group	Mean	95% CI	SD
Control	1.81	1.36-2.32	0.47
CD (group A)	25.88	22.38-29.30	3.57
CD + Colchicine-fenofibrate (group B)	20.94	18.63-23.10	2.90
CD + Colchicine-NAC (group C)	15.94	13.78-18.05	2.22

Table R18.6.1. Comparison of *KLF4* expression in rabbit thoracic aortas

ANOVA with Tukey's correction	*Mean Difference*	*95% CI of difference*	*p-value*
C vs CD	-24.07	-28.17 to -19.98	***<0.001***
C vs CD+Col+Fib	-19.13	-22.96 to -15.30	***<0.001***
C vs CD+Col+NAC	-14.13	-17.96 to -10.30	***<0.001***
CD vs CD+Col+Fib	4.94	1.11 to 8.77	***<0.001***
CD vs CD+Col+NAC	9.94	6.11 to 13.77	***<0.001***
CD+Col+Fib vs CD+Col+NAC	5.00	1.45 to 8.54	***<0.001***

Figure R18.5. Comparison of *KLF4* expression in rabbit thoracic aortas



C: control, CD: cholesterol diet (group A), Col: colchicine, Fib: fibrate, NAC: N-acetylcysteine

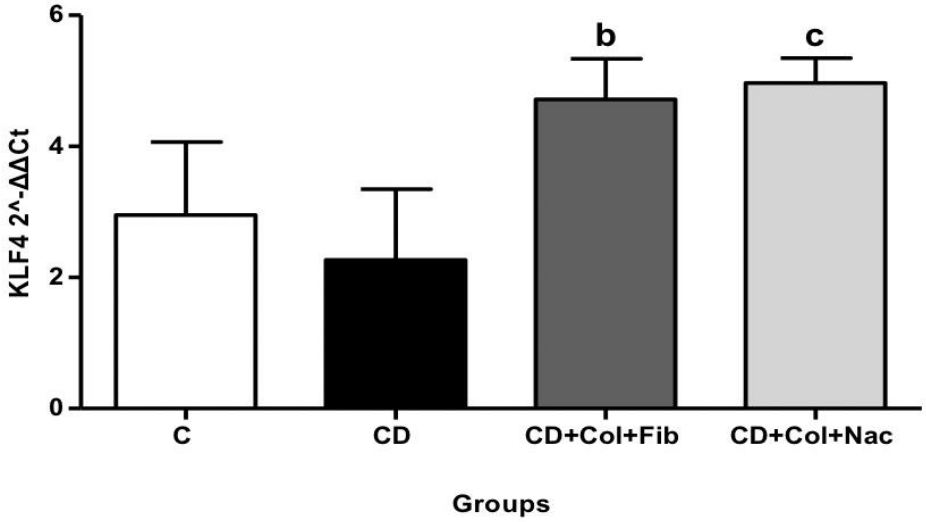
18.6.1 KLF4 in abdominal aortic specimens

The expression patterns of the *KLF4* gene in abdominal aortic specimens were different from those seen in the thoracic aorta. Particularly, no difference was observed in terms of *KLF4* expression between the control and the hypercholesterolemic group A. Study animals that received colchicine-based treatment experienced significantly greater abdominal *KLF4* levels compared to those that were subjected to hyperlipidemic diet without medical therapy. Colchicine regimens exerted similar effects on *KLF4* expression **(Table R18.6.2, Figure R18.6**).

Table R18.6.2. Comparison of *KLF4* expression in rabbit abdominal aortas

ANOVA with Tukey's correction	***Mean Difference***	***95% CI of difference***	***p-value***
C vs CD	0.68	-1.77 to 3.14	>0.05
C vs CD+Col+Fib	-1.77	-4.22 to 0.68	>0.05
C vs CD+Col+NAC	-2.01	-4.47 to 0.44	>0.05
CD vs CD+Col+Fib	-2.45	-4.65 to -0.25	***0.03***
CD vs CD+Col+NAC	-2.70	-4.90 to -0.50	***0.03***
CD+Col+Fib vs CD+Col+NAC	-0.24	-2.44 to 1.95	>0.05

Figure R18.6. Comparison of *KLF4* expression in rabbit abdominal aortas



18.7 BMP4 expression

18.7.1 BMP4 in thoracic aortic specimens

No statistically significant differences were observed in terms of *BMP4* expression in thoracic aortic specimens **(Table R18.7.1)**.

Table R18.7.1 Comparison of *BMP4* expression in rabbit thoracic aortas

ANOVA with Tukey's correction	*Mean Difference*	*95% CI of difference*	*p-value*
C vs CD	-2.16	-5.83 to 1.50	>0.05
C vs CD+Col+Fib	-0.79	-4.46 to 2.87	>0.05
C vs CD+Col+NAC	-1.41	-5.08 to 2.26	>0.05
CD vs CD+Col+Fib	1.37	-1.91 to 4.65	>0.05
CD vs CD+Col+NAC	0.75	-2.52 to 4.04	>0.05
CD+Col+Fib vs CD+Col+NAC	-0.61	-3.90 to 2.66	>0.05

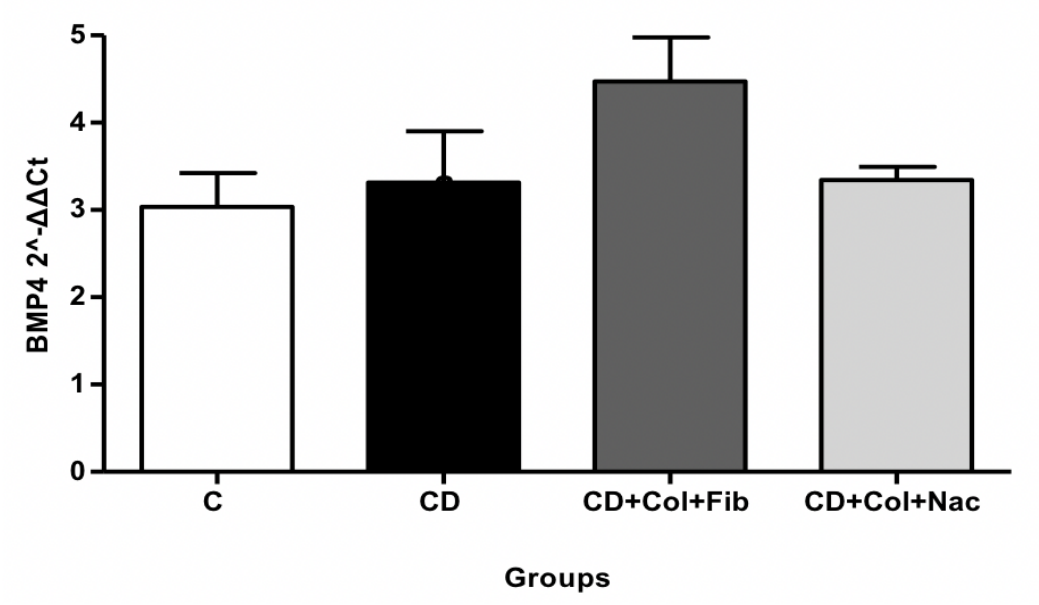
18.7.2 BMP4 in abdominal aortic specimens

The administration of hyperlipidemic diet and colchicine-based interventions also failed to affect *BMP4* expression in rabbit abdominal aortas **(Table R18.7.2, Figure R18.7**).

Table R18.7.2 Comparison of *BMP4* expression in rabbit abdominal aortas

ANOVA with Tukey's correction	*Mean Difference*	*95% CI of difference*	*p-value*
C vs CD	-0.28	-2.06 to 1.50	>0.05
C vs CD+Col+Fib	-1.44	-3.22 to 0.34	>0.05
C vs CD+Col+NAC	-0.31	-2.09 to 1.47	>0.05
CD vs CD+Col+Fib	-1.16	-2.94 to 0.62	>0.05
CD vs CD+Col+NAC	-0.03	-1.81 to 1.75	>0.05
CD+Col+Fib vs CD+Col+NAC	1.13	-0.65 to 2.91	>0.05

Figure R18.7. Comparison of *BMP4* expression in rabbit abdominal aortas



C: control, CD: cholesterol diet (group A), Col: colchicine, Fib: fibrate, NAC: N-acetylcysteine

18.8 SOX2 expression

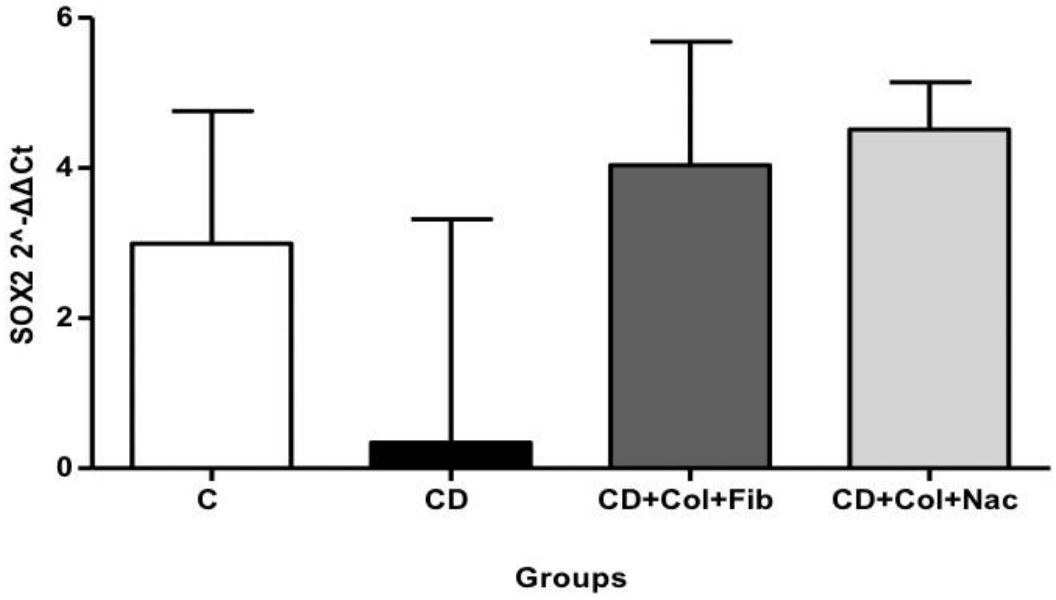
18.8.1 SOX2 in thoracic aortic specimens

No statistically significant differences were observed in terms of *SOX2* expression in thoracic aortic specimens **(Table R18.8.1, Figure R18.8**).

Table R18.8.1 Comparison of *SOX2* expression in rabbit thoracic aortas

ANOVA with Tukey's correction	*Mean Difference*	*95% CI of difference*	*p-value*
C vs CD	2.65	-2.43 to 7.73	>0.05
C vs CD+Col+Fib	-1.05	-6.13 to 4.03	>0.05
C vs CD+Col+NAC	-1.52	-6.60 to 3.55	>0.05
CD vs CD+Col+Fib	-3.70	-8.78 to 1.38	>0.05
CD vs CD+Col+NAC	-4.17	-9.25 to 0.90	>0.05
CD+Col+Fib vs CD+Col+NAC	-0.47	-5.55 to 4.60	>0.05

Figure R18.8. Comparison of *SOX2* expression in rabbit thoracic aortas



18.8.2 SOX2 in abdominal aortic specimens

The administration of hyperlipidemic diet and colchicine-based interventions again failed to affect *SOX2* expression in rabbit abdominal aortas **(Table R18.8.2**).

Table R18.8.2 Comparison of *SOX2* expression in rabbit abdominal aortas

ANOVA with Tukey's correction	*Mean Difference*	*95% CI of difference*	*p-value*
C vs CD	2.10	-5.97 to 2.26	>0.05
C vs CD+Col+Fib	2.37	-5.55 to 1.81	>0.05
C vs CD+Col+NAC	1.19	-4.62 to 2.74	>0.05
CD vs CD+Col+Fib	0.01	-4.13 to 4.10	>0.05
CD vs CD+Col+NAC	1.03	-3.20 to 5.03	>0.05
CD+Col+Fib vs CD+Col+NAC	1.17	-2.76 to 4.60	>0.05

C: control, CD: cholesterol diet (group A), Col: colchicine, Fib: fibrate, NAC: N-acetylcysteine

18.9 OCT4 expression

18.9.1 OCT4 in thoracic aortic specimens

OCT4 expression was similar in the thoracic aortas of the various animal groups that were assessed in the present experiment **(Table R18.9.1)**.

Table R18.9.1 Comparison of *OCT4* expression in rabbit thoracic aortas

ANOVA with Tukey's correction	*Mean Difference*	*95% CI of difference*	*p-value*
C vs CD	-2.87	-6.34 to 0.60	>0.05
C vs CD+Col+Fib	-1.03	-4.13 to 2.07	>0.05
C vs CD+Col+NAC	-2.38	-5.48 to 0.72	>0.05
CD vs CD+Col+Fib	1.84	-1.63 to 5.31	>0.05
CD vs CD+Col+NAC	0.48	-2.98 to 3.95	>0.05
CD+Col+Fib vs CD+Col+NAC	-1.35	-4.45 to 1.75	>0.05

18.9.2 OCT4 in abdominal aortic specimens

OCT4 expression in rabbit abdominal aortas was also unaffected by the administration of hypercholesterolemic diet and colchicine-based anti-inflammatory treatments **(Table R18.9.2**).

Table R18.9.2 Comparison of *OCT4* expression in rabbit abdominal aortas

ANOVA with Tukey's correction	*Mean Difference*	*95% CI of difference*	*p-value*
C vs CD	0.73	-3.44 to 4.90	>0.05
C vs CD+Col+Fib	0.24	-3.93 to 4.41	>0.05
C vs CD+Col+NAC	1.06	-3.11 to 5.23	>0.05
CD vs CD+Col+Fib	-0.49	-4.66 to 3.68	>0.05
CD vs CD+Col+NAC	0.32	-3.84 to 4.50	>0.05
CD+Col+Fib vs CD+Col+NAC	0.82	-3.35 to 4.99	>0.05

C: control, CD: cholesterol diet (group A), Col: colchicine, Fib: fibrate, NAC: N-acetylcysteine

18.10 NANOG expression

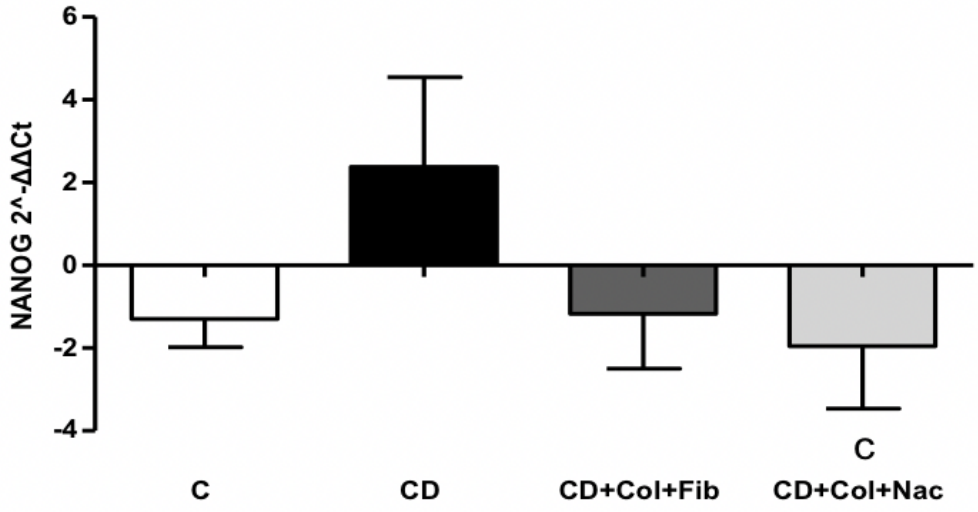
18.10.1 NANOG in thoracic aortic specimens

There was a trend towards thoracic *NANOG* upregulation in the unmedicated, hyperlipidemic group A compared to controls. That said, the threshold for statistical significance was not reached. Colchicine-based interventions curtailed the hyperlipidemia-driven upregulation of *NANOG* to some extent. Interestingly, *NANOG* was significantly reduced compared to group A only in the colchicine/NAC subgroup (MD: 4.33, 95% CI: 0.37 to 8.29, p=0.04), **(Table R18.10.1, Figure R18.9**).

Table R18.10.1 Comparison of *NANOG* expression in rabbit thoracic aortas

ANOVA with Tukey's correction	***Mean Difference***	***95% CI of difference***	***p-value***
C vs CD	-3.67	-7.64 to 0.28	0.06
C vs CD+Col+Fib	-0.12	-4.08 to 3.84	>0.05
C vs CD+Col+NAC	0.65	-3.30 to 4.62	>0.05
CD vs CD+Col+Fib	3.55	-0.40 to 7.51	0.06
CD vs CD+Col+NAC	4.33	0.37 to 8.29	***0.04***
CD+Col+Fib vs CD+Col+NAC	0.78	-3.18 to 4.74	>0.05

Figure R18.9. Comparison of *NANOG* expression in rabbit thoracic aortas



18.10.2 NANOG in abdominal aortic specimens

NANOG expression was similar in the abdominal aortas of the four study groups **(Table R18.10.2**).

Table R18.10.2 Comparison of *NANOG* expression in rabbit abdominal aortas

ANOVA with Tukey's correction	*Mean Difference*	*95% CI of difference*	*p-value*
C vs CD	1.62	-2.23 to 4.61	>0.05
C vs CD+Col+Fib	0.09	-3.01 to 3.12	>0.05
C vs CD+Col+NAC	2.41	-1.48 to 4.64	>0.05
CD vs CD+Col+Fib	1.54	-4.55 to 2.29	>0.05
CD vs CD+Col+NAC	0.53	-3.03 to 3.82	>0.05
CD+Col+Fib vs CD+Col+NAC	2.32	-1.54 to 4.58	>0.05

C: control, CD: cholesterol diet (group A), Col: colchicine, Fib: fibrate, NAC: N-acetylcysteine

18.11 NOTCH1 expression

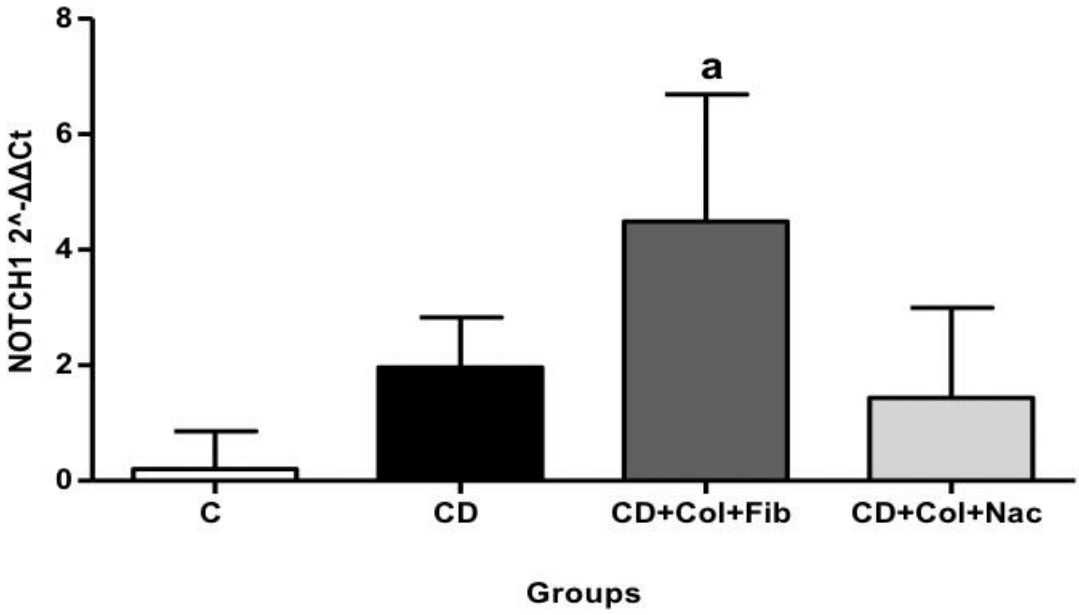
18.11.1 NOTCH1 in thoracic aortic specimens

Hypercholesterolemic diet alone did not significantly affect *NOTCH1* expression patterns in rabbit thoracic aortic specimens. Compared to controls, animals that underwent anti-inflammatory treatment with colchicine plus fenofibrate while being fed hyperlipidemic diet exhibited significant *NOTCH1* upregulation in their thoracic aortas (MD: -4.29, 95% CI: -8.09 to -0.48, p=0.03). Interestingly, the combination of colchicine with NAC did not result in a statistically notable increase in thoracic *NOTCH1* expression **(Table R18.11.1, Figure R18.10**).

Table R18.11.1 Comparison of *NOTCH1* expression in rabbit thoracic aortas

ANOVA with Tukey's correction	*Mean Difference*	*95% CI of difference*	*p-value*
C vs CD	-1.76	-5.56 to 2.04	>0.05
C vs CD+Col+Fib	-4.29	-8.09 to -0.48	***0.03***
C vs CD+Col+NAC	-1.24	-5.04 to 2.56	>0.05
CD vs CD+Col+Fib	-2.53	-6.33 to 1.27	>0.05
CD vs CD+Col+NAC	0.52	-3.28 to 4.32	>0.05
CD+Col+Fib vs CD+Col+NAC	3.05	-0.75 to 6.85	>0.05

Figure R18.10. Comparison of *NOTCH1* expression in rabbit thoracic aortas



C: control, CD: cholesterol diet (group A), Col: colchicine, Fib: fibrate, NAC: N-acetylcysteine

18.11.2 NOTCH1 in abdominal aortic specimens

NOTCH1 expression was similar in the abdominal aortas of the four study groups **(Table R18.11.2**).

Table R18.11.2 Comparison of *NOTCH1* expression in rabbit abdominal aortas

ANOVA with Tukey's correction	*Mean Difference*	*95% CI of difference*	*p-value*
C vs CD	-0.28	-6.48 to 5.92	>0.05
C vs CD+Col+Fib	-0.23	-6.43 to 5.97	>0.05
C vs CD+Col+NAC	1.07	-5.12 to 7.28	>0.05
CD vs CD+Col+Fib	0.05	-5.50 to 5.60	>0.05
CD vs CD+Col+NAC	1.36	-4.19 to 6.91	>0.05
CD+Col+Fib vs CD+Col+NAC	1.31	-4.24 to 6.86	>0.05

18.12 HIF1a expression

18.12.1 HIF1a in thoracic aortic specimens

HIF1a expression was similar in the thoracic aortas of the various animal groups that were assessed in the present experiment **(Table R18.12.1)**.

Table R18.12.1 Comparison of *HIF1a* expression in rabbit thoracic aortas

ANOVA with Tukey's correction	*Mean Difference*	*95% CI of difference*	*p-value*
C vs CD	2.52	-4.17 to 9.21	>0.05
C vs CD+Col+Fib	-1.38	-8.08 to 5.31	>0.05
C vs CD+Col+NAC	4.90	-1.79 to 11.60	>0.05
CD vs CD+Col+Fib	-3.90	-10.60 to 2.79	>0.05
CD vs CD+Col+NAC	2.38	-4.31 to 9.07	>0.05
CD+Col+Fib vs CD+Col+NAC	6.28	-0.41 to 12.98	>0.05

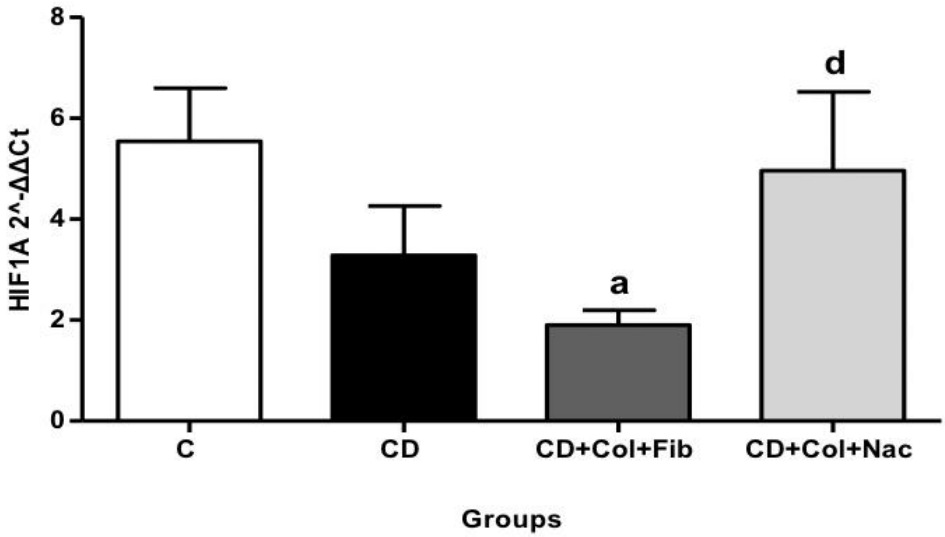
18.12.2 HIF1a in abdominal aortic specimens

HIF1a expression in the abdominal aortas of group B animals was significantly reduced compared to the controls (MD: 3.64, 95% CI: 0.83 to 6.44, p=0.03). No difference was observed in terms of abdominal aortic *HIF1a* when comparing the hypercholesterolemic group A to groups B and C **(Table R18.12.2, Figure R18.11**).

Table R18.12.2 Comparison of *HIF1a* expression in rabbit abdominal aortas

ANOVA with Tukey's correction	***Mean Difference***	***95% CI of difference***	***p-value***
C vs CD	2.26	-0.54 to 5.06	>0.05
C vs CD+Col+Fib	3.64	0.83 to 6.44	***0.03***
C vs CD+Col+NAC	0.58	-2.21 to 3.38	>0.05
CD vs CD+Col+Fib	1.38	-1.42 to 4.18	>0.05
CD vs CD+Col+NAC	-1.67	-4.47 to 1.12	>0.05
CD+Col+Fib vs CD+Col+NAC	-3.05	-5.85 to -0.25	***0.03***

Figure R18.11. Comparison of *HIF1a* expression in rabbit abdominal aortas



C: control, CD: cholesterol diet (group A), Col: colchicine, Fib: fibrate, NAC: N-acytelcysteine

18.13 HOXA5 expression

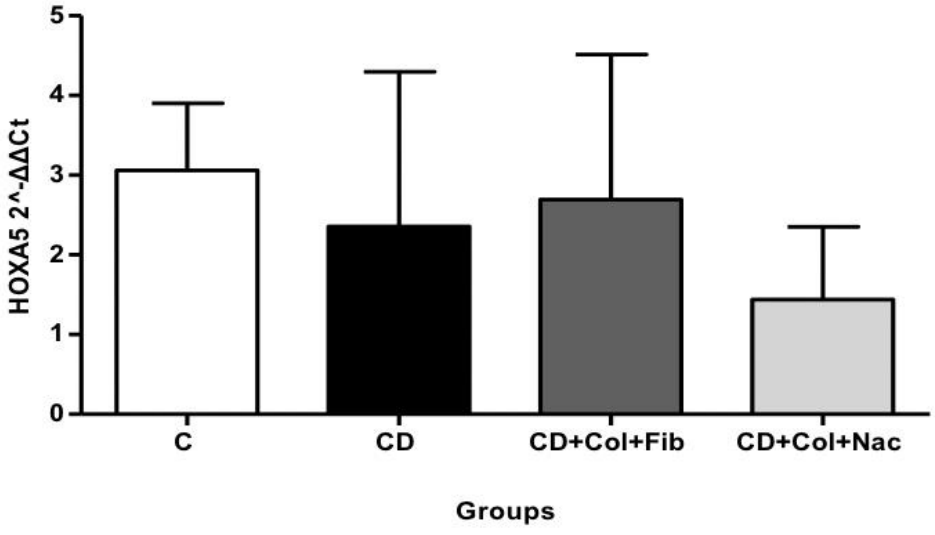
18.13.1 HOXA5 in thoracic aortic specimens

HOXA5 expression was similar in the thoracic aortas of the four study groups (**Table R18.13.1, Figure R18.12)**.

Table R18.13.1 Comparison of *HOXA5* expression in rabbit thoracic aortas

ANOVA with Tukey's correction	*Mean Difference*	*95% CI of difference*	*p-value*
C vs CD	0.70	-3.49 to 4.90	>0.05
C vs CD+Col+Fib	0.36	-3.38 to 4.11	>0.05
C vs CD+Col+NAC	1.62	-2.13 to 5.37	>0.05
CD vs CD+Col+Fib	-0.33	-4.53 to 3.85	>0.05
CD vs CD+Col+NAC	0.91	-3.28 to 5.11	>0.05
CD+Col+Fib vs CD+Col+NAC	1.25	-2.49 to 5.01	>0.05

Figure R18.12. Comparison of *HOXA5* expression in rabbit thoracic aortas



C: control, CD: cholesterol diet (group A), Col: colchicine, Fib: fibrate, NAC: N-acetylcysteine

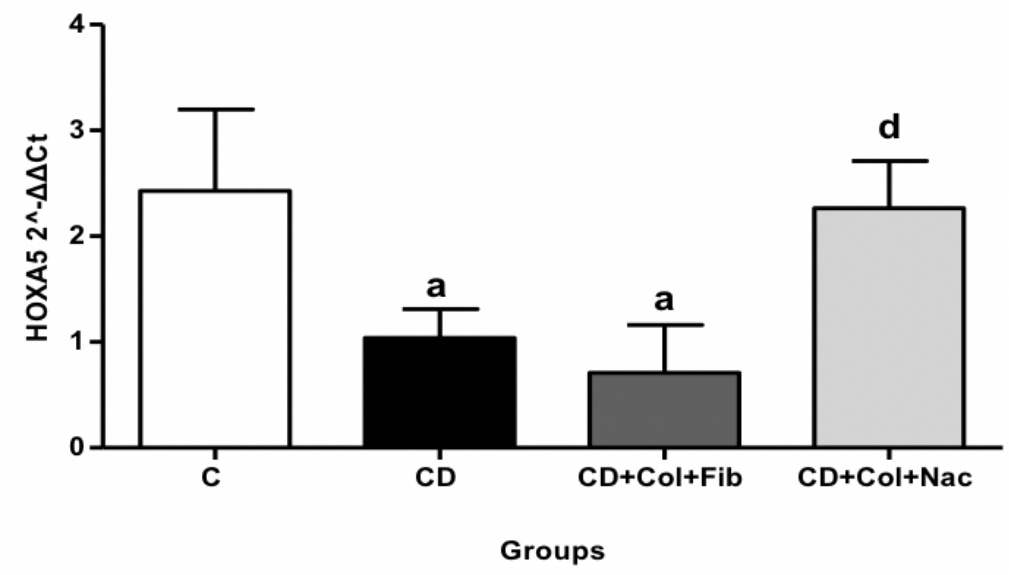
18.13.2 HOXA5 in abdominal aortic specimens

HOXA5 expression was reduced in group A animals compared to controls (MD: 1.39, 95% CI: 0.03 to 2.74, p=0.02). *HOXA5* remained downregulated in the setting of colchicine/fenofibrate treatment (MD: 1.71, 95% CI: 0.36 to 3.06, p=0.03). Interestingly, animals that received colchicine with NAC exhibited upregulation of *HOXA5* in their abdominal aortas back to the levels of controls **(Table R18.13.2, Figure R18.13)**.

Table R18.13.2 Comparison of *HOXA5* expression in rabbit abdominal aortas

ANOVA with Tukey's correction	***Mean Difference***	***95% CI of difference***	***p-value***
C vs CD	1.39	0.03 to 2.74	***0.03***
C vs CD+Col+Fib	1.71	0.36 to 3.06	***0.02***
C vs CD+Col+NAC	0.16	-1.19 to 1.51	>0.05
CD vs CD+Col+Fib	0.32	-1.02 to 1.67	>0.05
CD vs CD+Col+NAC	-1.23	-2.58 to 0.12	>0.05
CD+Col+Fib vs CD+Col+NAC	-1.55	-2.90 to -0.20	***0.03***

Figure R18.13. Comparison of *HOXA5* expression in rabbit abdominal aortas

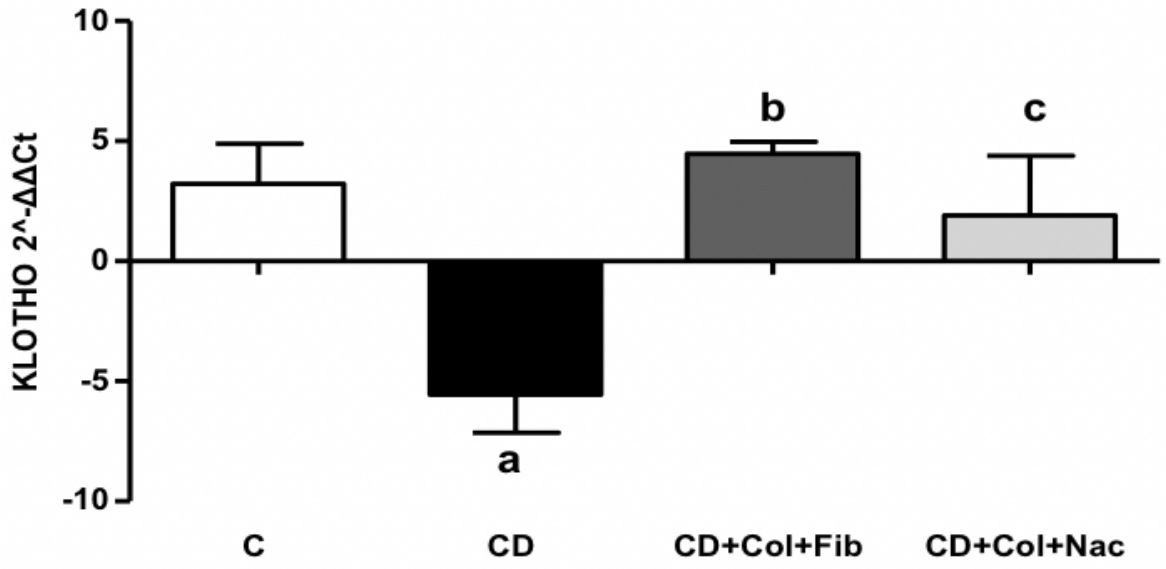


18.14 α-Klotho expression

18.14.1 α-Klotho in thoracic aortic specimens

Thoracic aortic *α-Klotho* expression was significantly reduced in the context of unmedicated hyperlipidemia (MD: 8.79, 95% CI: 1.82 to 15.76, p<0.001). Both colchicine/fenofibrate (MD: -10.04, 95% CI: -17.00 to 3.07, p<0.001) and colchicine/NAC (MD: -7.47, 95% CI: -13.83 to -1.11, p<0.001) led to significant upregulation of *α-Klotho* and effectively returned its expression back to baseline levels. No difference was observed between the two colchicine-based regimens in terms of thoracic aortic *α-Klotho* expression levels **(Table R18.14.1, Figure R18.14)**.

Figure R18.14. Comparison of *α-Klotho* expression in rabbit thoracic aortas



C: control, CD: cholesterol diet (group A), Col: colchicine, Fib: fibrate, NAC: N-acetylcysteine

Table R18.14.1 Comparison of *α-Klotho* expression in rabbit thoracic aortas

ANOVA with Tukey's correction	*Mean Difference*	*95% CI of difference*	*p-value*
C vs CD	8.79	1.82 to 15.76	***<0.001***
C vs CD+Col+Fib	-1.24	-8.21 to 5.72	>0.05
C vs CD+Col+NAC	1.31	-5.04 to 7.67	>0.05
CD vs CD+Col+Fib	-10.04	-17.00 to -3.07	***<0.001***
CD vs CD+Col+NAC	-7.47	-13.83 to -1.11	***<0.001***
CD+Col+Fib vs CD+Col+NAC	2.56	-3.79 to 8.92	>0.05

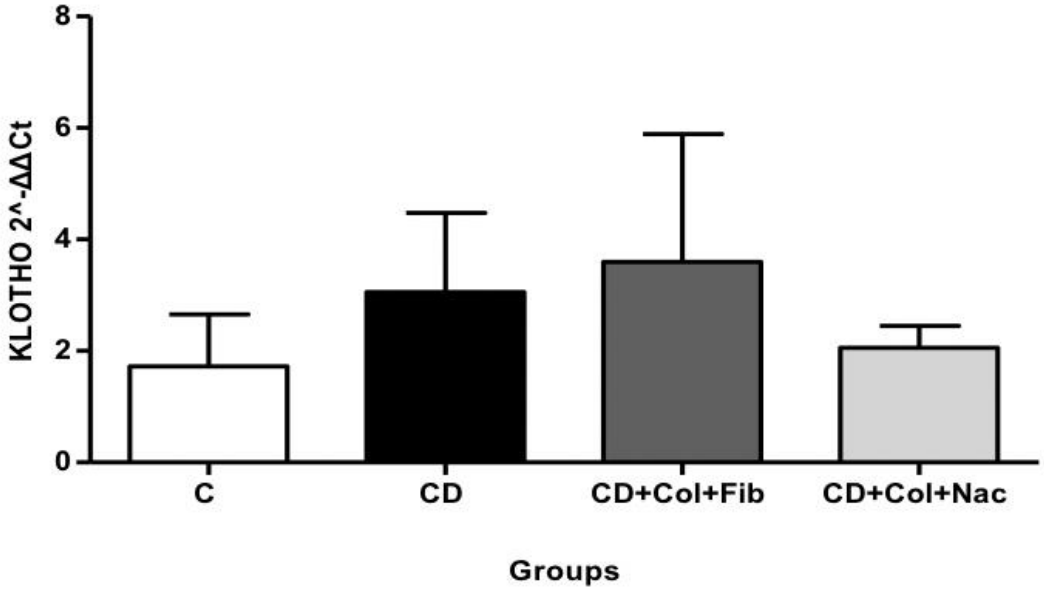
18.14.2 α-Klotho in abdominal aortic specimens

α-Klotho expression was similar in the abdominal aortas of the four study groups **(Table R18.14.2, Figure R18.15)**.

Table R18.14.2 Comparison of *α-Klotho* expression in rabbit abdominal aortas

ANOVA with Tukey's correction	*Mean Difference*	*95% CI of difference*	*p-value*
C vs CD	-1.33	-6.57 to 3.90	>0.05
C vs CD+Col+Fib	-1.87	-6.65 to 2.90	>0.05
C vs CD+Col+NAC	-0.34	-5.12 to 4.44	>0.05
CD vs CD+Col+Fib	-0.54	-5.32 to 4.23	>0.05
CD vs CD+Col+NAC	0.99	-3.78 to 5.77	>0.05
CD+Col+Fib vs CD+Col+NAC	1.53	-2.74 to 5.81	>0.05

Figure R18.15. Comparison of *α-Klotho* expression in rabbit abdominal aortas



C: control, CD: cholesterol diet (group A), Col: colchicine, Fib: fibrate, NAC: N-acetylcysteine

18.15 Summary of findings

18.15.1 Thoracic aorta

A synopsis of thoracic aortic findings is provided in ***Tables 15.1.1-15.1.3***.

Table R15.1.1 Comparing hyperlipidemic animals to controls in terms of thoracic aortic gene expression

Gene	*CD*	*CD+Col+Fib*	*CD+Col+NAC*
KLF4	***↑, p<0.001***	***↑, p<0.001***	***↑, p<0.001***
BMP4	ns	ns	ns
SOX2	ns	ns	ns
OCT4	ns	ns	ns
NANOG	ns	ns	ns
NOTCH1	ns	***↑, p<0.03***	ns
HIF1a	ns	ns	ns
HOXA5	ns	ns	ns
α-Klotho	***↓, p<0.001***	ns	ns

Table R15.1.2. Comparing hyperlipidemic animals receiving colchicine-based regimens to unmedicated hyperlipidemic animals in terms of thoracic aortic gene expression

Gene	*CD+Col+Fib*	*CD+Col+NAC*
KLF4	↓, ***p<0.001***	↓, ***p<0.001***
BMP4	ns	ns
SOX2	ns	ns
OCT4	ns	ns
NANOG	ns	↓, ***p=0.04***
NOTCH1	ns	ns
HIF1a	ns	ns
HOXA5	ns	ns
α-Klotho	↑, ***p<0.001***	↑, ***p<0.001***

Table R15.1.3. Comparing hyperlipidemic animals receiving colchicine/fenofibrate to colchicine/NAC in terms of thoracic aortic gene expression

Gene	*Colchicine-based regimens*
KLF4	↓NAC, ***p<0.001***
BMP4	ns
SOX2	ns
OCT4	ns
NANOG	ns
NOTCH1	ns
HIF1a	ns
HOXA5	ns
α-Klotho	ns

18.15.2 Abdominal aorta

A synopsis of abdominal aortic findings is provided in ***Tables 15.2.1-15.2.3***.

Table R15.2.1 Comparing hyperlipidemic animals to controls in terms of abdominal aortic gene expression

Gene	*CD*	*CD+Col+Fib*	*CD+Col+NAC*
KLF4	ns	ns	ns
BMP4	ns	ns	ns
SOX2	ns	ns	ns
OCT4	ns	ns	ns
NANOG	ns	ns	ns
NOTCH1	ns	ns	ns
HIF1a	ns	***↓, p=0.03***	ns
HOXA5	***↓, p=0.03***	***↓, p=0.02***	ns
α-Klotho	ns	ns	ns

Table R15.2.2. Comparing hyperlipidemic animals receiving colchicine-based regimens to unmedicated hyperlipidemic animals in terms of abdominal aortic gene expression

Gene	*CD+Col+Fib*	*CD+Col+NAC*
KLF4	***↑, p=0.03***	***↑, p=0.03***
BMP4	ns	ns
SOX2	ns	ns
OCT4	ns	ns
NANOG	ns	ns
NOTCH1	ns	ns
HIF1a	ns	ns
HOXA5	ns	ns
α-Klotho	ns	ns

Table R15.2.3. Comparing hyperlipidemic animals receiving colchicine/fenofibrate to colchicine/NAC in terms of abdominal aortic gene expression

Gene	*Colchicine-based regimens*
KLF4	ns
BMP4	ns
SOX2	ns
OCT4	ns
NANOG	ns
NOTCH1	ns
HIF1a	ns
HOXA5	↑**NAC, *p=0.03***
α-Klotho	ns

Figure R18.16. Statistically significant associations in group A

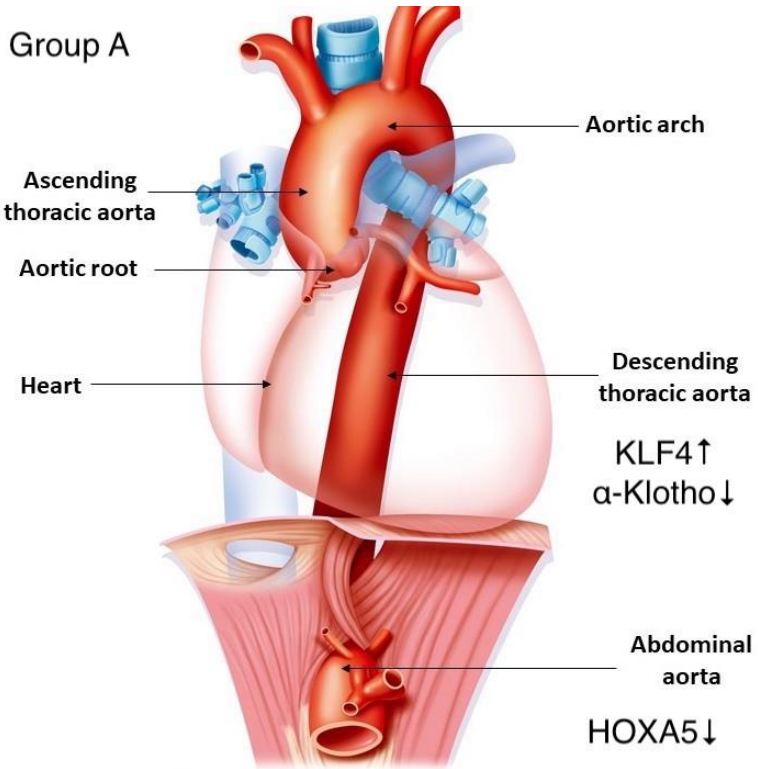


Figure R18.17. Statistically significant associations in group B

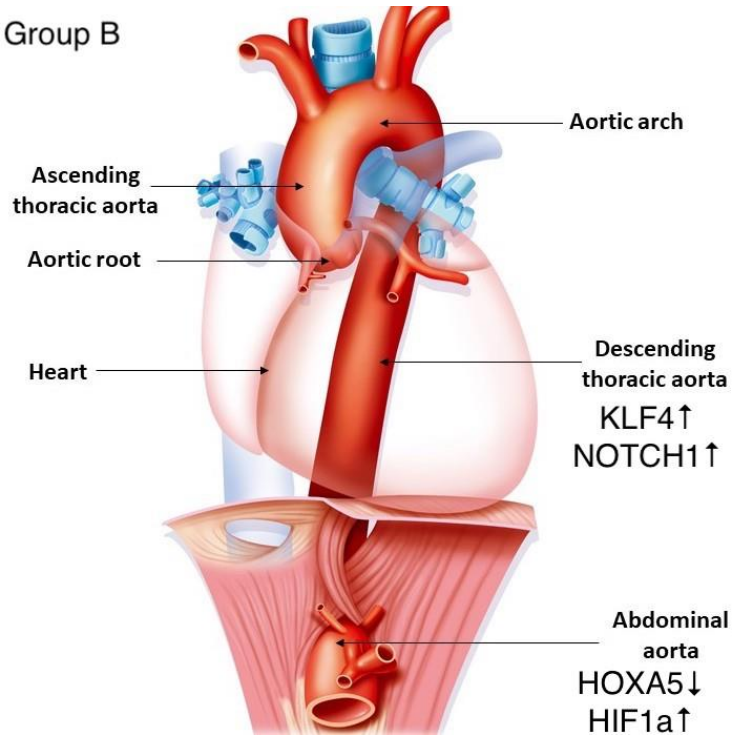
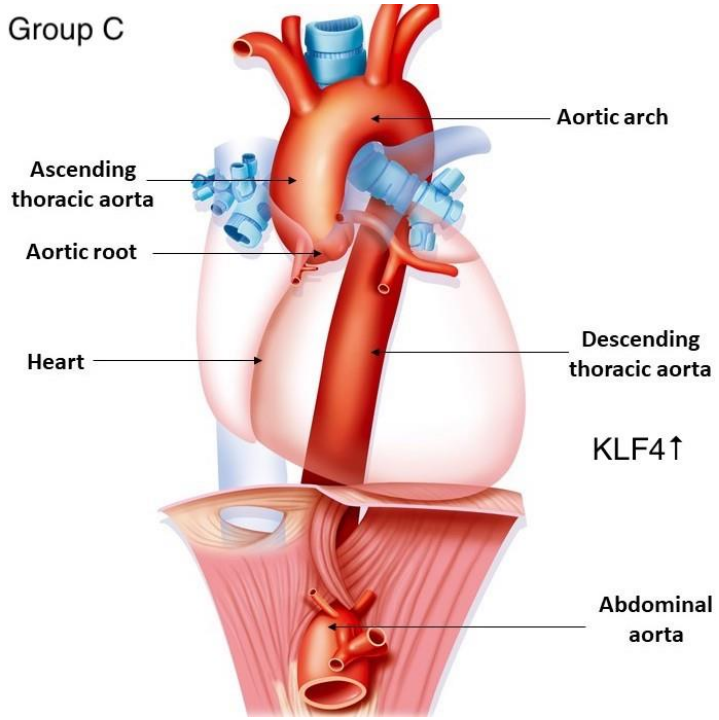


Figure R18.18. Statistically significant associations in group C



CHAPTER 19

Discussion

In recent years, numerous landmark studies have affirmed the instrumental role that inflammation plays in atherogenesis.[4, 245] A growing body of literature has also shown that dysregulation of stem cell gene expression can occur due to shear stress-related inflammation. This genetic derangement appears to orchestrate the development and progression of atherosclerosis.[245]

Several attempts have been made to utilize anti-inflammatory agents and immunomodulators as tools against atherogenesis.[18] To begin with, the seminal LoDoCo and COLCOT trials found that low dose colchicine significantly reduces the risk of cardiovascular death not only in the setting of stable CAD but also in acute coronary syndromes.[72, 73] From a pathophysiology standpoint, colchicine suppresses the activation of inflammasome NLRP3 in neutrophils and macrophages by inhibiting tubulin polymerization and microtubule generation. As a result, the production of inflammatory cytokines, such as IL1b and IL18, is curtailed. [70, 71]

Furthermore, compelling evidence suggests that fibrates bear atheroprotective properties beyond their conventional hypolipidemic action.[76, 77] As PPARα agonists, fibrates, effectively block NF-kB thereby halting the expression of inflammatory genes, including those coding adhesion molecules (i.e., VCAM1, ICAM1), matrix modulators (i.e. MMP9), and tissue factor.[78] Fenofibrate, in particular, directly inhibits the production of interleukins (including IL-6 and IL-

1β) and COX2 (which is expressed by endothelial cells, smooth muscle cells, and macrophages in atherosclerotic lesions).[76, 79]

Although formally not indicated as an atheroprotective agent, NAC attenuates proatherogenic inflammation due to its antioxidant effects.[80] Indeed, NAC scavenges ROS and other free radicals that trigger and propagate inflammatory stimuli. NAC also induces structural changes in the TNF-α receptor and inhibits the activation of NF-κB. Furthermore, NAC hinders adipose tissue differentiation via downregulation of MAPKs (i.e phospho-ERK and phospho-JNK).[80] In addition, it downregulates PPARγ by affecting CEBP/β expression.[246] NAC also reduces LDL oxidization, obstructs triglyceride accumulation in adipose cells, and diminishes the number and size of foam cells.[247] Interestingly, oral NAC supplementation has been shown to decrease CRP and IL-6 plasma levels in a non-linear fashion.[81]

In the present experimental study, we sought to delineate the impact of colchicine-based anti-inflammatory regimens on curtailing the *de novo* development of thoracic and abdominal aortic atheromatosis on a histological, biochemical, and genetic level. Benchmark animal models have shown that four to twelve weeks of hyperlipidemic feeding are adequate for the formation of atherosclerotic lesions. [230, 235] To that end, cholesterol-enriched chow was fed to white New Zealand rabbits for seven weeks. As previously described, group A animals were fed hyperlipidemic diet alone, group B animals were fed the same diet enriched with colchicine and fenofibrate, while group C animals were fed hypercholesterolemic diet with the addition of colchicine and NAC. In line with institutionally validated protocols [235], we administered all medication *per os* to avoid eliciting injection-related stress which could potentially affect inflammatory markers, including the expression of developmental genes.

At baseline, body weights and serum biochemical findings were similar among all animals. The administration of 1% (w/w) cholesterol diet for seven weeks produced the desired model, as evidenced by a significant increase in body weights, plasma total cholesterol and LDL levels as well as by the presence of typical atherosclerotic lesions in rabbit aortas. Although, colchicine dosing (2 mg/kg/day) was higher compared to previous animal trials, no diarrhea or cases of weight loss were noted during the course of the experiment.[240] Colchicine also had no adverse effect on liver and renal function.

By the end of the experiment, HDL values were significantly higher in animals that received colchicine-based treatment compared to baseline and T1. Furthermore, colchicine interventions reduced triglycerides significantly compared to baseline levels and to hyperlipidemic diet alone. The addition of fenofibrate to colchicine produced a more robust reduction in triglycerides compared to NAC. This is not surprising considering that fibrates are well known for their role in regulating TG levels. The hypolipidemic effect of colchicine is likely multifactorial. First, recent data have indicated that colchicine may function similarly to niacin by promoting apolipoprotein B (apoB) breakdown thereby diminishing VLDL formation. Second, certain groups have suggested that colchicine may act as an antagonist of the bile acid receptor farnesoid X receptor, thus mimicking the lipid-lowering action of guggulsterone.[248, 249] Others have also hypothesized that inhibition of acyl CoA/diacylglycerol acyltransferase (DGAT-1) using small-molecules (such as colchicine) may induce a potent reduction in triglyceride concentrations – (DGAT1 is one of two known DGAT enzymes that catalyze the final step in triglyceride biosynthesis). [250]

As previously explained, all hyperlipidemic groups developed atheromatosis on a macroscopic and histologic level. Although atherosclerotic lesions did not regress down to the

levels of controls, both groups receiving anti-inflammatory therapy experienced significantly less thoracic and abdominal aortic atheromatosis compared to their unmedicated hyperlipidemic counterparts. Combining colchicine with NAC instead of fibrate resulted in stronger atheroprotection both in the thoracic and the abdominal aorta.

In athero-prone regions, low shear stress forces cause inappropriate activation of developmental signaling pathways, leading to increased inflammation and vascular permeability.[101] In light of this phenomenon, we sought to explore the impact of prolonged hyperlipidemia as well as the effect of anti-inflammatory therapy on a variety of atheroprotective (*KLF4, Klotho, HOXA5, NOTCH1, OCT4*) and proatherogenic (*HIF1a, SOX2, BMP4, NANOG*) genes.

KLF4 was significantly upregulated in the thoracic aortas of all hypercholesterolemic groups despite being essentially latent in controls. Of note, thoracic *KLF4* overexpression was less pronounced in colchicine arms. Importantly, the addition of NAC restricted thoracic *KLF4* expression more extensively compared to fenofibrate. These trends were likely an effect of reduced proatherogenic inflammation rather than the sequalae of an interaction of the medication with the *KLF4* gene itself.

On the other hand, the *KLF4* gene followed a different expression pattern in abdominal aortas. In contrast to its thoracic counterpart, *KLF4* was not latent in the abdominal aortas of controls. As a matter of fact, no difference was identified in terms of abdominal KLF4 levels between controls and the hypercholesterolemic group A. Study animals that received colchicine-based treatment experienced significant abdominal *KLF4* upregulation compared to those that were subjected to hyperlipidemic diet alone. Interestingly, colchicine regimens exerted similar effects on abdominal *KLF4* expression. Although to a different extent, *KLF4* was ultimately

upregulated both in the thoracic and in the abdominal aortas of hyperlipidemic animals receiving colchicine-based treatment.

Many theories have been proposed to explain the protective role that KLF4 exhibits against vascular insult. First, hemodynamically disturbed flow induces *KLF4* expression which leads to vasodilation through the upregulation of endothelial NO synthase.[251] Second, KLF4 induces VE-cadherin transcription, elicits the release of thrombomodulin, and inhibits TNF-mediated expression of cellular attachment molecules such as VCAM-1 and E-selectin [124, 127] KLF4 also attaches to three predicted KLF consensus binding sites in the Connexin 40 (Cx40) promoter. Of note, both VE-cadherin and Cx40-mediated gap junctional communication favor endothelial homeostasis.[252] Lastly, KLF4 inhibits NF-kB and interacts with STAT6 to promote macrophage/monocyte differentiation to the anti-inflammatory M2 phenotype instead of its pro-inflammatory M1 counterpart. [253]

Several other agents, including statins, have also been shown to inhibit proatherogenic inflammation through KLF4-mediated cascades.[254] For example, resveratrol, a natural phenol found in grapes and certain berries, has antithrombotic properties due to its MEK5/MEF2-dependent induction of *KLF4* in endothelial cells.[255, 256] Similarly, lunasin and tannic acid which are natural peptides found in soybean seeds, soy-derived food products, and other grains act upon the KLF2/4 cascade via MEK5/ERK, degrade oxidized-LDL and inhibit monocyte adhesion on endothelial cells.[122, 123] Surprisingly, there is also data suggesting that vorinostat, a histone deacytalase inhibitor used to treat cutaneous T cell lymphoma, activates *KLF2* and *KLF4* via myocyte enhancer factor 2 and may be helpful in severe atherosclerosis [257]

Klotho expression was significantly reduced in the atherosclerotic thoracic aortas of animals receiving hyperlipidemic diet alone compared to controls. Both colchicine/fenofibrate and

colchicine/NAC led to significant upregulation of *α-Klotho* and effectively elevated its expression back to baseline levels. No statistically significant difference was observed between the two colchicine-based regimens in terms of thoracic aortic *α-Klotho* expression levels.

Klotho is crucial in maintaining vascular homeostasis. Downregulation of its expression has been strongly associated not only with atheromatosis, but also with aging and advanced chronic kidney disease.[218] An extensive body of literature has confirmed the association of Klotho with maintenance of endothelial integrity, reduction of oxidative stress, and endothelial NO production.[220] Additionally, Klotho reduces the expression of the intramembrane calcium channel TRPC6, which when expressed in VSMCs, favors the development of arterial wall remodeling, calcification, and ultimately leads to atherosclerosis and hypertension.[227]

Animals lacking Klotho have increased expression of the type III cotransporters PiT-1 and PiT-2 which mediate phosphate-induced VSMC calcification.[223-225] Moreover, Klotho deficiency induces the osteogenic transcription factor CBFA1/RUNX2 in VSMCs which further promotes vascular calcification. On the other hand, the addition of Klotho to VSMCs *in vitro*, decreases phosphate uptake by suppressing the activity of type III cotransporters and prevents the phenotypic switch of VSMCs to an osteochondrogenic phenotype.[223] Importantly, both systemic and local inflammation have been associated with reduced renal Klotho levels. Of note, neutralization of inflammatory cytokines has been shown to reverse Klotho expression.[222, 258] Our study is the first build upon that experience by recording the recovery of Klotho levels in atherosclerotic aortic tissue.

Colchicine/fenofibrate also significantly upregulated thoracic *NOTCH1* compared to controls. The addition of NAC to colchicine failed to produce the same result. Thus, the upregulation of *NOTCH1* was likely an effect of fenofibrate. Unfortunately, the present study was

not designed to elucidate the mechanism behind this important observation. Therefore, future research is warranted to better understand whether PPRAα agonists (like fibrates) interact with NOTCH1 via conventional or non-canonical signaling.

Nevertheless, it should be emphasized that part of the atheroprotective effect of colchicine/fenofibrate in thoracic aortas was due to *NOTCH1* upregulation. Importantly, NOTCH1 safeguards endothelial and junctional integrity.[183] It also hinders the expression of pro-inflammatory cytokines and adhesion molecules.[182] On the other hand, downregulation of *NOTCH1* due to low shear stress promotes cellular proliferation by inducing the overexpression of cell cycle regulators (i.e., cyclins and CDKs). Reduced levels of NOTCH1 also lead to between-cell instability due to upregulation of genes involved in intracellular calcium homeostasis such as CAMK2B and Apelin.[184] Connexin-37 and aconitase levels also increase in the absence of NOTCH1 - which in turns leads to defective gap junctions.[187]

Combining colchicine with NAC significantly reduced thoracic *NANOG* expression compared to hyperlipidemic diet alone. A growing body of literature has attributed proatherogenic properties to NANOG. First, it promotes osteopontin upregulation and VSMC phenotypic switch. Overexpression of *NANOG* also enhances the proliferation, migration, and anti-apoptosis capabilities of vascular SMCs.[171] Lastly, *NANOG* upregulation has been shown to result in the loss of VE-cadherin from adherens junctions. [172] All things considered, the downregulation of *NANOG* contributed to the anti-atherogenic effect of colchicine/NAC on thoracic aortas.

In the abdominal aorta, hypercholesterolemic diet resulted in significant downregulation of the atheroprotective *HOXA5* gene. Reduced *HOXA5* expression has been implicated in intimal hyperplasia and derailed angiogenesis.[210] HOXA5 downregulation also promotes proatherogenic gene expression, extracellular matrix modification, and integrin alterations.[209]

In the setting of HOXA5 deficiency macrophage cells and VSMCs switch towards the proinflammatory M1 phenotype.[209]

The combination of colchicine plus NAC reversed HOXA5 levels back to baseline whereas the coadministration of colchicine with fenofibrate failed to do so. Of note, *HOXA5* upregulation promotes AJ stabilization by increasing the retention of beta-catenin. This process diminishes vascular permeability.[205] HOXA5 also maintains endothelial integrity by regulating specific genes (i.e., thrombospondin-2, vascular endothelial growth factor receptor-2, ephrin-A1, HIF1a, prostaglandin-endoperoxide synthase-2, and PTEN).[208]

In abdominal aortas, colchicine/fenofibrate downregulated the proatherogenic *HIF1a* gene compared to baseline. Indeed, low hemodynamic shear stress induces *HIF1a* expression in response to localized endothelial hypoxia. HIF1a levels are also driven by 1) NF-κB-mediated overproduction and 2) cezanne-mediated de-ubiquitination which salvages HIF1a from proteasomal degradation.[197]

HIF1α drives atherogenesis not only by promoting intraplaque angiogenesis but also by triggering the production of adhesion molecules such as CXCL1, ICAM1, and VCAM1.[198, 199] Furthermore, HIF1α promotes inflammation and cellular proliferation in low shear stress conditions by inducing the expression of several enzymes involved in the metabolism of glucose (PFKFB3, hexokinase 2 & enolase 2, glucose transporters 1 and 3).[108] In addition, HIF1α-induced upregulation of *KLF4* promotes migration of VSMCs thereby increasing the size of atherosclerotic lesions.[200] HIF1α also promotes the phenotypic switch of macrophages and monocytes towards the M1 pro-inflammatory phenotype.[201] The inhibition of the aforementioned processes via colchicine/fenofibrate accounted for at least part of this regimen's atheroprotective effect on rabbit abdominal aortas.

Last but not least, no statistically significant differences were noted in terms of the osteogenic *BMP4*, *SOX2,* and *OCT4* genes in thoracic and abdominal aortic specimens.[132, 160] This is not surprising considering that no calcified atherosclerotic lesions developed in any of the groups during this seven-week experiment. Indeed, the lack of variation in osteogenic gene expression is likely an implication of the relatively short study period rather than an actual effect of anti-inflammatory treatment.

The present study has certain limitations that should be acknowledged. Firstly, study animals were distributed randomly yet unequally in four treatment arms. The control and the unmedicated hypercholesterolemic group A each included six animals, while groups B and C which underwent colchicine-based treatment each comprised of eight animals. Abiding by the 3R paradigm (Replacement, Reduction, Refinement), our goal was to minimize the number of animals used in our experiment.[259] Second, we did not include a colchicine-only group on account of prior research suggesting that colchicine alone may not be enough to halt *de novo* atherogenesis in hyperlipidemic rabbits.[235] Third, our study was not designed to delineate the exact pathways through which stem cell genes moderate atherogenesis. Lastly, cellular staining using stem cell markers was not performed due to scarce resources.

Moving forward, our lab aims to investigate the biomechanics of aortic atheromatosis. Indeed, the thoracic and abdominal aorta differ in terms of length, diameter, curvatures, and branch network. These features affect intrinsic hemodynamics and shear stress forces[260, 261], and likely account for the variations in atherosclerotic burden and gene dysregulation that were noted in our experiment. Last but not least, we plan to explore the correlation between the aortic expression of stem cell genes and corresponding serum levels in atheromatosis (since these are more convenient to track clinically).

CHAPTER 20

Conclusions

- Colchicine-based anti-inflammatory regimens curtailed the *de novo* development of thoracic and abdominal aortic atheromatosis in rabbits receiving hyperlipidemic diet.
- Combining colchicine with NAC instead of fenofibrate attenuated thoracic and abdominal aortic atherogenesis more robustly.
- The expression pattern of aortic stem cell genes was spatially influenced by western-type diet and could be modified using colchicine-based anti-inflammatory regimens.
- Hyperlipidemic diet drove *de novo* thoracic and abdominal aortic atherogenesis by downregulating *α-Klotho* and *HOXA5*, respectively.
- Both colchicine regimens halted thoracic aortic atheromatosis by upregulating *α-Klotho*. In the thoracic aorta, combining colchicine with fenofibrate also increased *NOTCH1*, while the addition of NAC reduced *NANOG*.
- In the abdominal aorta, combining colchicine with fenofibrate reduced *HIF1a*, whereas the addition of NAC upregulated *HOXA5*.
- Last but not least, the atheroprotective *KLF4* gene was upregulated both in the thoracic and in the abdominal aortas of hyperlipidemic animals receiving colchicine-based treatment.

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