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# Relations between aortic valve calcification and telomere length dynamics

Ilona Saraieva

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Mention: «Sciences de la Vie et de la Santé»

par **Ilona SARAIEVA**

# Implication de la longueur des télomères dans le processus de calcification des valves aortiques

le 12 Novembre 2020

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***To those, whom I love***

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# Abbreviations

8-oxodG – 8-oxo-7,8-dihydro-2'-deoxyguanosine

AKT – protein kinase B

ALP – alkaline phosphatase

AS – aortic atenosis

$\alpha$ SMA – alpha smooth muscle actin

BICD1 – protein bicaudal D homolog 1 gene

BMP2 – bone morphogenetic protein 2

BRG1 – SWI/SNF-related chromatin remodelling protein

CACNA1C – calcium voltage-gated channel subunit alpha1 C

CAVD – calcific aortic valve disease

CTC1 – CST telomere replication complex component 1

DCAF4 – damage specific DNA binding protein 1 and cullin-4A associated factor 4

DCK1 – dyskerin

DDX11 – DEAD/H-box helicase 11

DNMT3A – DNA methyltransferase 3 alpha

DZ – dizygotic

ECM – extracellular matrix

eNOS – endothelial nitric oxide synthase

ENPP1 – ectonucleotide pyrophosphatase/pyrophosphorylase

EPC – endothelial progenitor cells

GATA5 – GATA binding protein 5

GWAS – genome-wide association study

HIF-1 $\alpha$  – hypoxia inducible factor 1 $\alpha$

IL-1 $\beta$  – interleukin 1 $\beta$

IL-6 – interleukin 6

ISG15 – interferon stimulated gene 15 kDa

ITAS – internal telomerase assay standard

kb – kilobase

KD – Kolmogorov distance

Lp(a) – lipoproteine (a)

Lp-PLA2 – lipoprotein-associated phospholipase A2

LTL – leukocyte telomere length

MGP – matrix Gla protein

MMP – matrix metalloproteinases

MZ – monozygotic

NAF1 – nuclear assembly factor 1 ribonucleoprotein

NF- $\kappa$ B – nuclear factor kappa B

NHP2 – H/ACA ribonucleoprotein complex subunit 2

NOP10 – H/ACA ribonucleoprotein complex subunit 3

NOTCH1 – neurogenic locus notch homolog protein 1

OBFC1 – oligonucleotide/oligosaccharide-binding fold containing 1

OxPLs – oxidized phospholipids

PARN – poly(A)-specific ribonuclease

POT1 – protection of telomeres protein 1

PPi – inorganic pyrophosphate

qFISH – quantitative fluorescence *in situ* hybridization

qPCR – quantitative polymerase chain reaction

RAP1 – repressor/activator protein 1  
ROS – reactive oxygen species  
RTEL1 – regulator of telomere elongation  
helicase 1  
RUNX2 – runt-related transcription factor 2  
SASP – senescent associated phenotype  
SDS – sodium dodecyl sulfate  
SNP – single-nucleotide polymorphism  
SSC – saline sodium citrate  
STELA – single TL analysis  
TCBA1 – T-cell lymphoma breakpoint-  
associated target 1 gene  
TERC – telomerase RNA component  
TERT – telomerase reverse transcriptase  
TeSLA – telomere shortest length assay

TGF- $\beta$ 1 – transforming growth factor beta  
TIMP– tissue inhibitor of metalloproteinase  
TIN2 – TRF1-interacting protein 2  
TL – telomere length  
TNF- $\alpha$  – tumor necrosis factor alpha  
TRF – telomeric restriction fragments  
TRF1 – telomeric repeat binding factor 1  
TRF2 – telomeric repeat binding factor 2  
TTP1 – POT1-TIN2 organizing protein  
U-STELA – universal STELA  
VEC – valvular endothelial cells  
VEGF – vascular endothelial growth factor  
VIC – valvular interstitial cells  
ZNF676 – zinc finger protein 676 gene

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# Résumé français

## Situation du sujet

La sténose (ou rétrécissement) aortique (SA) est la maladie cardiaque valvulaire la plus courante dans les pays développés. Elle se caractérise par un rétrécissement progressif et l'obstruction de l'ouverture de la valve aortique en raison de l'épaississement des feuillets valvulaires aortiques, ce qui entrave l'écoulement du sang du ventricule gauche et induit des modifications pathologiques dans celui-ci [1]. Il s'agit d'une maladie liée à l'âge et son incidence est estimée à environ 20 cas pour 1000 dans la population âgée de plus de 65 ans [2,3]. En raison de la hausse de l'espérance de vie, une augmentation de la prévalence de la sténose aortique est prévue dans les prochaines années [4]. On considère que la sténose aortique présente des facteurs de risque communs et une pathogenèse similaire à celle de l'athérosclérose. Ainsi, les principaux facteurs de risque du développement de la SA sont en plus de l'âge avancé, le sexe masculin et un taux élevé de Lipo-protéine (a) plasmatique [3]. Parmi les autres facteurs de risque, il convient de mentionner l'hypertension [3], l'obésité [5], le diabète de type 2 [6], le tabagisme [3,7], le syndrome métabolique [8,9] et l'insuffisance rénale chronique [10]. En effet, le développement de ces deux maladies (SA et athérosclérose) commence par une lésion locale de l'endothélium, suivie d'une accumulation de lipides, d'une infiltration de cellules immunitaires et d'une inflammation chronique de faible intensité. Dans les tissus de la valve aortique, ces événements déclenchent la transdifférenciation des cellules interstitielles valvulaires (CIV), le remodelage de la matrice extracellulaire, la mort cellulaire programmée et la calcification.

Il est important de noter que la SA se produit avec une prévalence identique dans les valves tricuspides et les valves bicuspidées [11]. Actuellement, il n'existe pas de traitement médicamenteux efficace de la SA, et l'unique façon de la traiter est l'implantation d'une valve aortique prothétique. Sans intervention chirurgicale, les patients atteints de SA grave

ont un mauvais pronostic avec un taux de mortalité pouvant atteindre 50 % dans l'année [12].

Récemment, de nombreux mécanismes cellulaires et moléculaire du vieillissement ont été associés à l'apparition et à la progression de la sténose aortique [13]. Il est suggéré que certains de ces mécanismes moléculaires pourraient être induits par des télomères courts [14]. Les télomères sont les extrémités des chromosomes, ils raccourcissent avec les divisions cellulaires au cours du vieillissement et lorsqu'ils atteignent une taille critique, ils déclenchent l'arrêt de la prolifération connue comme la sénescence réplivative. Les cellules sénescents favorisent le dysfonctionnement des tissus et l'inflammation chronique par leur phénotype de sécrétion et participent donc au développement de maladies liées à l'âge [14,15]. En outre, il a été constaté, avant même la sénescence réplivative, que la longueur des télomères avaient un effet sur l'expression des gènes par des interactions spatiales dans la chromatine, ou effet de position télomérique (EPT) [16].

Par ailleurs, il a été démontré que les leucocytes de patients souffrant d'un rétrécissement aortique présentent des télomères plus courts que ceux de sujets sains [17]. Enfin, une étude récente sur un modèle murin a montré que la longueur des télomères était susceptible d'influencer l'expression de gènes dérégulés dans les valves calcifiées [18].

Nous émettons donc l'hypothèse que la longueur des télomères dans les valves pourrait jouer un rôle dans l'apparition et la progression de la SA à travers le phénomène de calcification. Les personnes ayant des télomères plus courts présenteraient ainsi un risque plus élevé de développer un rétrécissement aortique.

### **Objectifs du travail**

L'objectif général de ce travail est de déterminer la relation entre la dynamique des télomères dans les valves aortiques et le processus de calcification. Plus précisément, les objectifs de l'étude sont les suivants :

- I. Décrire les caractéristiques générales de la dynamique des télomères dans les valves aortiques humaines en utilisant une analyse transversale.
- II. Étudier la dynamique des télomères dans les valves aortiques humaines chez des individus avec et sans SA.
- III. Explorer localement la dynamique des télomères dans différentes zones de valves calcifiées.

La dynamique des télomères dans les valves est évaluée par la longueur moyenne des télomères, la longueur des télomères les plus courts, la forme de distribution des longueurs des télomères et l'activité de la télomérase.

### **Méthodologie générale**

Dans cette étude, nous avons utilisé 88 valves prélevées sur des patients ayant subi une chirurgie de remplacement valvulaire pour une sténose aortique sévère, une régurgitation valvulaire aortique ou une dilatation aortique. Des valves tricuspides et bicuspidées ont été utilisées dans l'étude. Les patients ont été divisés en 2 groupes : ceux souffrant de sténose aortique ont été inclus dans le groupe SA, tandis que les patients diagnostiqués avec une régurgitation aortique ou une dilatation aortique ont été inclus dans le groupe non-SA. De plus, en fonction de la présence ou non de signes macroscopiques de calcification sur la valve, le groupe non-SA a été subdivisé en patients présentant des valves sclérotiques et non sclérotiques.

Chaque valve a fait l'objet d'une évaluation macroscopique pour détecter la présence de calcification. Sur la base de cette évaluation, les valves aortiques ont été disséquées en : i) zones normales (zones lisses sans calcifications) ; ii) zones épaissies (zones fibrotiques sans présence évidente de calcifications) et iii) zones calcifiées (zones avec calcifications macroscopiques) comme décrit précédemment [19]. Après que notre analyse préliminaire n'a pas montré de différence entre la longueur des télomères dans les zones normales et

les zones épaissies, nous avons regroupé ces deux catégories sous le terme zones "non calcifiées". En outre, un sous-groupe de valves sans calcification macroscopique a été disséqué selon les zones anatomiques, à savoir la pointe et la base de la valve.

Parmi les 88 valves, 84 ont été collectées pour les mesures de la longueur des télomères (LT) et 4 pour les tests d'activité de la télomérase. Seize de ces patients ont été exclus de l'étude en raison de la quantité et/ou de la qualité insuffisantes de l'ADN extrait des tissus pour les mesures de la LT. Ainsi, seules les valves de 72 patients ont été utilisées dans l'étude (68 pour la mesure de la LT et 4 pour l'activité télomérase). Parmi les 68 patients avec mesures de la LT, des données supplémentaires d'analyse transcriptomique ont été obtenues pour un sous-groupe de 8 patients.

La longueur des télomères a été mesurée par la méthode de Southern blot. Cette méthode permet l'obtention de la longueur moyenne des télomères. De plus, grâce à un traitement mathématique du signal de Southern blot nous avons pu mesurer à chaque prélèvement valvulaire la distribution des longueurs des télomères (DLT) et la longueur des télomères les plus courts. L'activité de la télomérase dans les extraits protéiques de tissus a été réalisé par test TRAP (Telomere Repeat Amplification Protocol). L'analyse des profils d'expression génique a été effectuée par analyse transcriptomique sur puce des ARN extraits des tissus.

Deux types de comparaisons ont été effectuées : des comparaisons inter-individuelles entre les différents groupes de patients et des comparaisons intra-individuelles (intra-valve) entre différentes zones de la valve d'un même individu.

## **Résultats obtenus**

Dans les zones non-calcifiées des valves, on observe une tendance à la diminution de la LT avec l'âge. En revanche, aucune association de la LT n'a été mise en évidence avec le sexe ou le type de valve (bicuspide/tricuspide).

Dans les valves calcifiées, les zones calcifiées présentent des télomères nettement plus courts que les zones non calcifiées (n=31,  $8.58 \pm 0.73$  kb vs  $8.12 \pm 0.75$  kb,  $p < 0.0001$ ), alors qu'aucune activité télomérase n'a été détectée dans l'une ou l'autre des parties de la valve. La différence moyenne de LT entre les deux zones était de 462 bases. Aucune différence n'a été constatée entre les zones normales et les zones épaissies, ni entre la pointe et la base des valves. En outre, les patients du groupe SA ont des télomères plus courts dans les zones non calcifiées de leur valve que ceux du groupe de non-SA ( $8.40 \pm 0.64$  kb vs  $8.85 \pm 0.65$ ,  $p = 0.01$ ). L'analyse de la DLT a révélé que la calcification, au-delà de la LT moyenne, est associée à des changements dans la forme de la DLT. Enfin, l'analyse des données de transcriptomique en composantes principales a démontré que, indépendamment de la présence de calcification, les tissus ayant des télomères courts ont un profil d'expression génique différent par rapport aux tissus avec des télomères longs.

## **Discussion**

Dans cette thèse, nous avons étudié la dynamique des télomères dans des valves aortiques au cours de la SA. Les quatre principaux résultats de ce travail sont les suivants: 1) les patients diagnostiqués avec une SA ont des télomères plus courts dans les zones non-calcifiées par rapport aux individus sans SA; 2) les valves avec des calcifications présentent des télomères plus courts dans leurs zones calcifiées que dans les zones non-calcifiées; 3) les valves avec des télomères plus courts présentent un profil transcriptomique distinct de celles avec des télomères plus longs; 4) la calcification et la SA ont un impact sur la distribution de la LT dans les valves. Pris ensemble, ces résultats suggèrent que les télomères jouent un rôle complexe dans la progression de la maladie. D'une part, les télomères courts pourraient participer à l'apparition et la progression de la SA. D'autre part, le processus de calcification semble entraîner localement une diminution supplémentaire de la LT dans les zones calcifiées

### ***Dynamique des télomères dans les valves aortiques***

Dans cette première étude visant à étudier la LT directement dans le tissu valvulaire, nous avons observé une tendance à la diminution de la LT dans les zones non calcifiées avec l'âge. Le rythme de raccourcissement calculé est égal à 15 paires de bases par an, ce qui est similaire à l'attrition télomérique dans les muscles squelettiques, un tissu peu prolifératif [20]. De plus, la LT dans les zones non-calcifiées des valves pour toute la cohorte était de  $8,59 \pm 0,68$  kb, ce qui est proche de la LT dans les muscles de patients du même âge dans l'étude TELARTA (n=259, LT du muscle:  $8,54 \pm 0,72$  kb ; âge:  $63 \pm 14$  ans) [20].

Nous n'avons observé aucune association entre la TL et le sexe, ce qui peut s'expliquer par le nombre relativement faible de femmes dans l'étude. La LT dans les valves était également indépendante de la présence ou pas de bicuspidie. Il est à noter que, dans notre étude, la proportion de valves tricuspides était plus élevée dans le groupe non-SA, bien qu'en moyenne, les personnes ayant des valves bicuspidies subissent des chirurgies de remplacement de la valve aortique avec une fréquence identique à celle des personnes ayant des valves tricuspides [11]. Cette différence de proportion est due à la sélection des échantillons pour les différentes expériences. Cependant, comme nous n'avons pas observé de différence de LT entre les individus ayant des valves bicuspidies et tricuspides, ce biais de sélection des échantillons ne semble pas avoir affecté nos résultats.

### ***Rôle de l'activité de la télomérase dans la dynamique des télomères des valves***

Nous n'avons pas détecté d'activité télomérase dans les tissus valvulaires lors de nos analyses. Ces résultats concordent avec les études qui montrent que, chez l'humain, l'activité de la télomérase est réprimée dans la plupart des cellules, à l'exception des cellules germinales, des cellules cancéreuses, des cellules souches et des lymphocytes activés. Cependant, cela n'est pas en accord avec de récentes études préliminaires, qui démontrent la présence de télomérase dans des CIV pendant la calcification [21], ou des

études plus anciennes qui montrent la présence de télomérase dans les plaques athérosclérotiques [22,23]. Cette différence peut s'expliquer par la différence d'approche méthodologique. Les résultats qui montrent la présence de télomérase dans les plaques d'athérome sont basés sur l'immunohistochimie qui ne détecte que la présence de la télomérase, alors que nous avons utilisé la méthode TRAP, qui mesure directement son activité. Il existe 22 isoformes de la télomérase chez l'humain et une seule de ces isoformes possède une activité d'élongation des télomères [24]. La grande majorité des tissus humains pourraient exprimer des variants inactifs de la télomérase [25]. Par conséquent, nous ne réfutons pas la présence potentielle d'isoformes inactives de télomérase dans les valves aortiques calcifiées [26] et plaques d'athérome [22,23] qui pourraient être impliquées dans des voies non canoniques de la télomérase.

### ***Le processus de calcification à l'origine des télomères courts observés dans les zones calcifiées des valves***

Dans les valves calcifiées, nous avons constaté que les zones calcifiées présentent des télomères plus courts que les zones non-calcifiées. Cette différence pourrait soit résulter d'un effet direct de la calcification, soit être présente dans les valves avant la maladie.

Pour étudier la relation temporelle entre les télomères courts et la calcification, nous avons évalué la LT dans des différentes zones anatomiques de valves macroscopiquement exemptes de calcification. On suppose que les nodules calcifiés apparaissent d'abord aux endroits où des forces hémodynamiques et biomécaniques accrues agissent sur la valve [27], à savoir à la base du cuspide, puis se propagent vers la pointe [28]. L'absence de différence de LT entre ces zones soutient l'idée que les télomères courts dans les lésions calcifiées de la valve aortique pourraient être un effet secondaire du processus de calcification. Il convient de noter que ces résultats sont en contradiction avec des études antérieures, qui montrent que les tissus vasculaires exposés à une contrainte hémodynamique plus importante ont des télomères plus courts que ceux qui sont soumis à

une contrainte de cisaillement moindre [29,30]. Cependant, on sait peu de choses sur la LT initiale dans ces différentes régions de l'arbre vasculaire, ainsi que sur l'effet du stress hémodynamique sur l'attrition des télomères.

L'écart moyen observé entre les zones non-calcifiées et calcifiées des valves était de 462 bases. Un calcul basé sur l'estimation du taux annuel d'attrition dans les valves suggère que la différence de LT entre les zones calcifiées et non-calcifiées est équivalente à au moins 30 ans "d'effet de l'âge". Si nous supposons que les télomères courts observés dans les zones calcifiées sont une conséquence du processus de calcification, ils peuvent provenir de l'attrition locale des télomères et/ou de l'infiltration des cellules immunitaires.

L'attrition locale des télomères pourrait être causée par un stress oxydant massif et une inflammation chronique [31]. En effet, les lésions calcifiées de la valve aortique présentent une capacité antioxydante diminuée, une génération d'espèces réactives de l'oxygène (ERO) augmentée ainsi qu'une l'infiltration des cellules immunitaires [32–36]. Il a été démontré que le stress oxydant et l'inflammation chronique provoquent une attrition des télomères *in vitro* [37,38] et *in vivo* [39–41] par la génération des ERO. Les ERO sont connues pour induire une oxydation et des cassures de l'ADN dans les séquences des télomères beaucoup plus fréquemment que dans d'autres séquences d'ADN [42,43]. En outre, les ERO ont de multiples effets sur les systèmes biologiques, comme l'oxydation de l'ADN, qui peuvent avoir une influence indirecte sur la maintenance des télomères [37,44], entraînant une mauvaise réparation et une perte de télomères. Nos résultats sont conformes à des études antérieures sur les tissus vasculaires humains qui ont signalé que les plaques athérosclérotiques, dont le développement est associé à une inflammation chronique et à un stress oxydatant, ont des télomères plus courts que les vaisseaux sains [29,41,45].

Dans notre étude, l'hypothèse d'une attrition locale de la LT par le stress oxydant est encore renforcée par l'absence de différence de LT constatée entre les zones normales et épaissies des valves. Bien que l'épaississement du tissu valvulaire aortique soit l'une des



étapes pathologiques qui précèdent la calcification [46], les zones épaissies sont caractérisées par une expression accrue de la superoxyde dismutase 3 [47]. Cette enzyme antioxydante peut prévenir les lésions de l'ADN dans les CIV [36] et ainsi prévenir le raccourcissement des télomères dans les tissus valvulaires épaissis. L'attrition télomérique se produirait alors au cours des étapes suivantes de la calcification qui sont caractérisées par un stress oxydant et une inflammation accrue [28,32,35].

Outre le stress oxydant, l'inflammation chronique entraîne une infiltration abondante de cellules immunitaires dans les lésions [28,32]. Ainsi, les zones calcifiées, en plus des cellules résidentes de la valve, comprennent des infiltrats inflammatoires [28,32]. Par conséquent, la LT mesurée dans le tissu complet de différentes zones des valves aortiques reflète leur composition cellulaire. En tenant compte d'études antérieures sur les tissus humains, qui ont montré que la LT dans les cellules sanguines est plus courte que dans d'autres tissus somatiques [20,48,49], nous supposons que les cellules immunitaires ont des télomères plus courts que les cellules résidentes de la valve aortique. Ainsi, l'infiltration abondante de cellules immunitaires dans les zones calcifiées de la valve peut partiellement contribuer à l'observation de télomères plus courts. À l'appui de cette hypothèse, nous n'avons observé aucune différence entre la LT dans les zones normales et épaissies des valves, qui sont moins infiltrées par les cellules immunitaires que les zones calcifiées [28,32].

### ***Une LT courte précède la SA et participe au développement de la calcification***

Dans cette étude, nous avons constaté que les patients atteints de SA présentent des télomères plus courts dans les zones non-calcifiées de leur valve que ceux des sujets sans SA. Cela suggère que des télomères courts seraient présents chez ces personnes indépendamment du développement de la maladie. Nous supposons que les télomères courts peuvent précéder la calcification de la valve et donc contribuer à l'apparition et à la progression de la maladie. En effet, il a été proposé que les télomères courts ne sont pas

seulement un biomarqueur majeur du vieillissement biologique [50] mais aussi une cause primaire de celui-ci [14]. Parmi les altérations cellulaires associées à la présence de télomères courts, on trouve la sénescence réplivative [14]. Lorsque les cellules sénescents s'accumulent, elles favorisent les changements liés à l'âge dans les tissus au travers de la régulation paracrine des cellules voisines par leur phénotype sécrétoire [14,15]. Les facteurs libérés par les cellules sénescents augmentent l'inflammation chronique, remodelent la matrice extra-cellulaire, ont un impact sur la capacité de régénération et contribuent ainsi aux changements fonctionnels des tissus et au développement de maladies liées à l'âge [14,15].

Avant même de déclencher la sénescence réplivative, il a également été démontré que les télomères peuvent modifier l'expression des gènes par l'effet de position télomérique (EPT) et l'EPT sur de longues distances [51]. Un nombre croissant d'études moléculaires ont démontré l'importance de la LT dans la régulation de l'expression des gènes [16,52–54]. Par exemple, en utilisant des cellules ayant une LT différente, Lou et ses collègues ont vérifié l'impact de la LT sur l'expression du gène 15 stimulé par l'interféron (ISG15) dans les fibroblastes humains [55]. Dans cette étude, le raccourcissement des télomères a conduit à une régulation positive de l'ISG15 [55], qui code pour ISG15, une protéine de type ubiquitine qui est un composant de la voie de réponse de l'immunité innée. L'auteur a suggéré qu'ISG15 pourrait contribuer à l'inflammation liée à l'âge et donc être associée aux maladies cardiovasculaires [55]. Des études ultérieures ont révélé un large spectre de gènes, dont l'expression est régulée par la LT [16,52–54]. Parmi eux, on trouve des gènes qui codent pour des protéines, qui pourraient être potentiellement impliquées dans la calcification de la valve aortique, comme BMP6 (protéine osseuse morphogénétique 6) et FOXC1 (forkhead box C1) [16,19,56]. De plus, une étude récente sur des souris a montré que les gènes dérégulés dans les valves calcifiées sont susceptibles d'être régulés de manière LT-dépendante [18]. Dans notre étude, nous avons utilisé l'analyse transcriptomique pour démontrer que, quel que soit le niveau de calcification, le profil d'expression diffère entre les tissus valvulaires ayant des télomères longs et courts. Ainsi, nous supposons que la LT en

elle-même peut contribuer partiellement au développement de la SA par la régulation de l'expression des gènes.

### ***Implication potentielle de la LT dans d'autres cellules dans le développement de la SA***

Des cellules non-résidentes du tissu valvulaire pourraient participer au lien entre LT courte et SA. En effet, il a été démontré précédemment que la LT intra-individuelle à travers les tissus somatiques est comparable [48,57,58]. Cela signifie que les sujets ayant des télomères courts (par rapport à leurs pairs) dans un tissu somatique présentent aussi généralement des télomères relativement courts dans d'autres tissus somatiques [48,57,58]. On peut donc supposer que les patients atteints de SA présentent des télomères courts à la fois dans les valves et d'autres cellules. Cette hypothèse est confirmée par l'étude de Kurz et al [59], qui a démontré la présence de télomères plus courts dans les leucocytes de patients atteints de SA. Récemment, une nouvelle hypothèse a été proposée pour expliquer l'implication d'une longueur des télomères des leucocytes (LTL) courte dans l'athérogénèse [60]. Les individus présentant une LTL courte ont une prévalence plus élevée d'hématopoïèse clonale [61]. L'hématopoïèse clonale correspond à une expansion clonale d'une cellule souche hématopoïétique unique qui devient alors sur-représentée parmi les cellules du sang périphérique. Ce phénomène augmente progressivement avec le vieillissement, est associé à un doublement du risque de maladies cardiovasculaires et à une stimulation des voies pro-inflammatoires [62]. Des clones de cellules T ont été retrouvés infiltrés dans des lésions calcifiées de la valve aortique [63,64]. De plus, l'hématopoïèse clonale est induite par des mutations somatiques dans des gènes récurrents des cellules souches hématopoïétiques [65,66] et certaines de ces mutations, en particulier dans les gènes TET2 et DNMT3A, ont été associées à une mortalité accrue chez les patients atteints de SA subissant une implantation de prothèse aortique par transcathétérisme [67]. Il est suggéré que la présence de ces mutations contribue à une

sécrétion accrue de cytokines pro-inflammatoires dans les cellules du sang périphérique telles que les cellules T et les monocytes [67].

Un autre mécanisme pour expliquer l'association entre la LT courte et la SA serait que des télomères plus courts dans le sang périphérique et/ou dans le tissu valvulaire pourraient également refléter des télomères plus courts dans les cellules progénitrices endothéliales (CPE), qui présentent une capacité de réparation endothéliale. La capacité de régénération de ces cellules peut être limitée celles ayant des télomères plus courts [68]. En effet, des résultats récents de notre équipe montrent qu'une LTL courte est associée à une LT-CPE courte ainsi qu'à un nombre réduit de CPE auto-renouvelables (données non publiées). La diminution de la capacité de réparation endothéliale des CPE pourrait jouer un rôle dans l'altération de la guérison des lésions endothéliales dans la première étape de développement de la SA [27,69]. Ainsi, des télomères plus courts dans les leucocytes, les progéniteurs endothéliaux et les valves pourraient jouer un rôle multiple et complexe dans le développement et la progression de la SA. Malheureusement, une limite de cette étude est que nous n'avons pas eu accès aux prélèvements sanguins des patients de la cohorte, ce qui aurait pu nous permettre de vérifier cette hypothèse.

### ***Rôle des télomères les plus courts dans la progression de la maladie et approches méthodologiques***

Dans une cellule, le raccourcissement à une longueur critique d'un petit sous-ensemble de télomères est suffisant pour provoquer un arrêt de la réplication [70], qui à son tour entraîne tout le spectre des changements cellulaires liés à la senescence et contribue au développement de maladies liées à l'âge. Par conséquent, dans ce travail, nous nous sommes concentrés non seulement sur la LT moyenne mais aussi sur la longueur des télomères les plus courts de la distribution. Nous avons trouvé des différences au niveau des télomères les plus courts entre les zones non-calciifiées et calciifiées ainsi qu'entre ceux des sujets atteints de SA et ceux des sujets non atteints de SA, alors qu'aucune différence

n'a été observée entre les tissus non-calcifiés, à savoir i) les zones anatomiques et ii) les zones valvulaires normales et épaissies. Ces résultats reflètent nos conclusions obtenues avec la LT moyenne, ce qui donne plus de crédit à l'implication d'un mécanisme biologique lié aux télomères les plus courts dans le développement de la SA.

### ***Changements dans la DLT de la valve pendant le développement de la SA***

L'analyse des distributions de la longueur des télomères dans les tissus et de leurs changements de forme pourrait fournir des informations supplémentaires sur la dynamique des télomères pendant la progression de la maladie. Il a déjà été démontré dans les leucocytes que même des personnes ayant la même LT moyenne peuvent avoir des formes de DLT différentes, ce qui signifie qu'elles présentent une proportion différente de télomères longs, moyens et courts [71]. Il a également été démontré que malgré un raccourcissement des télomères avec l'âge, la forme de la DLT dans les leucocytes est stable dans le temps [71]. Néanmoins, nous ne connaissons pas le profil de la DLT dans les tissus. Ici, nous avons montré que les formes des DLT sont très similaires dans différentes zones des valves non-sclérotiques du même sujet, alors que la forme des DLT varie beaucoup entre les individus de la cohorte étudiée. Ceci est proche du concept de signature de la DLT dans les leucocytes (stabilité de la forme dans le temps), mais cette "signature tissulaire" est une stabilité de la forme de la DLT dans l'espace (différentes zones d'un même tissu). Nous avons ensuite observé une perte progressive de cette "signature tissulaire" de la forme de la DLT de la valve au cours du processus de calcification. Nous suggérons que les modifications de la DLT valvulaire peuvent être causées par l'infiltration des cellules immunitaires et/ou le stress oxydant. En effet, l'infiltration dans les zones calcifiées de cellules ayant probablement une LT plus courte [20,48,49], et donc une DLT déplacée vers des télomères courts, provoquera un changement de forme de la DLT du tissu. De plus, avec la méthode de TRF, il a été démontré qu'un stress oxydant induit par les rayonnements ionisants peut modifier la distribution des télomères longs, moyens et courts

dans les cellules endothéliales des veines ombilicales humaines [72]. Nous avons constaté des modifications de la forme de la DLT dans les zones calcifiées des valves de patients atteints de sclérose aortique et de sténose aortique, mais l'ampleur des changements était similaire dans ces deux groupes. Nous pensons que cela pourrait s'expliquer par l'inflammation plus importante aux premiers stades du développement de la SA par rapport aux stades terminaux, qui sont caractérisés par une calcification accélérée [73,74]. Par conséquent, les changements établis de la forme de la DLT chez les patients atteints de sclérose aortique se maintiendraient chez les patients atteints de SA sans empirer.

Il est intéressant de noter que, bien que nous ayons observé des changements intra-valvulaires de la forme des DLT au cours du développement de la SA, ces changements étaient toujours plus faibles que les variations interindividuelles de forme de DLT dans l'ensemble de la population. Une analyse plus approfondie des variations interindividuelles de la forme des DLT a révélé que les personnes atteintes de SA pourraient présenter plus de similitudes de forme des DLT dans les zones non-calcifiées entre elles qu'avec les patients non atteints de SA. Si ces résultats étaient confirmés, il s'agirait d'une première suggestion d'une DLT caractéristique pour les individus plus enclins à développer une SA. Toutefois, étant donné la faible différence entre les valeurs médianes, le nombre élevé d'observations et le niveau de significativité statistique limite, il faudrait confirmer ce résultat par une autre approche de calcul. Si nous supposons qu'il n'y a pas de différences entre les variabilités des formes de DLT entre les groupes, c'est une indication que la prédisposition à développer la SA n'est pas liée à une forme/répartition particulière de la DLT mais plutôt à la présence de télomères courts. Il est à noter que lorsque les distances de Kolmogorov sont calculées pour les comparaisons inter-valves, le nombre de distances obtenues est égal à  $n*(n-1)/2$ , où  $n$  est le nombre d'échantillons dans le groupe. Par conséquent, l'apparition de valeurs aberrantes peut affecter la puissance statistique.

En résumé, l'utilisation de nouvelles approches de calcul pour étudier les formes de DLT élargit notre compréhension de la dynamique des télomères et ouvre de nouvelles

possibilités d'interprétation de ses liens avec des processus pathologiques tels que la calcification des valves.

### **Conclusions générales**

Dans ce travail de thèse, nous avons étudié l'implication de la dynamique des télomères dans la SA et la calcification des valves aortiques. Nos résultats suggèrent que l'approfondissement des connaissances sur la biologie des télomères pourrait fournir des indices pour mieux comprendre la pathogénie des maladies cardiovasculaires. Ici, nous avons observé une relation bilatérale entre la LT et la progression de la SA dégénérative. D'une part, les télomères courts pourraient être impliqués dans l'apparition et le développement de la SA et d'autre part, le raccourcissement local des télomères dans les valves aortiques pourrait être causé par des mécanismes impliqués dans le processus de calcification.

Dans ce contexte, les études futures devraient accorder plus d'attention aux aspects moléculaires de la dynamique des télomères et leur implication dans la pathogénèse de la SA.

# Foreword

Aortic stenosis (AS), one of the major causes of heart failure, is often related to progressive valve calcification. The prevalence of AS increases with age and approximates up to 20 people per 1000 in a population aged 65 years and over. Currently, there is no effective drug therapy to reduce the progression of AS and the only treatment for severe AS is aortic valve replacement. Deciphering the molecular mechanisms of valve calcification and discovering new parameters or risk factors that influence the progression of the disease can be of great interest to clinical practice that would allow better patient care.

Telomeres are nucleoprotein structures at the ends of linear chromosomes that maintain genomic integrity. Telomere length (TL) shortens with aging, and numerous studies have used TL as a biomarker of “biological aging”. The credence to TL as a biomarker of aging has been established, showing that short TL is found in a vast range of age-related diseases (neurodegenerative, metabolic, and cardiovascular diseases). However, recent knowledge on telomere dynamics led to the hypothesis that short TL should not be considered as a consequence of aging and a biomarker of age-related disease progression but rather as a determinant and an independent risk factor of these diseases.

Experimental evidence suggests that mechanisms of valvular calcification involve aging pathways that might be activated by short telomeres. It was demonstrated that patients with AS have shorter telomeres in white blood cells in comparison to healthy matched controls. However, the role of telomeres in aortic valve tissue remains unknown.

We hypothesize that short telomeres may play a role in the onset and progression of AS.

The objective of this thesis is to provide evidence for a better understanding of the role of telomeres in AS and particularly in the calcification process. To this end we studied telomere dynamics in valve tissue of patients with or without AS and in different areas of aortic valves with different stages of calcification.



# Introduction

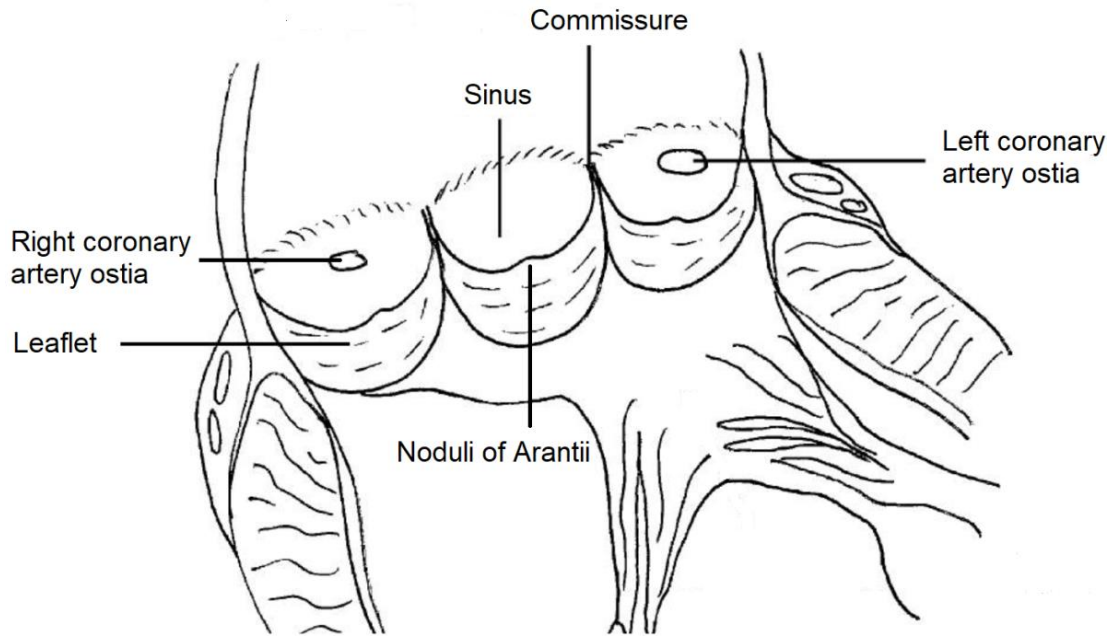
## 1 Pathophysiology of aortic valve stenosis

### 1.1 Aortic valve structure

The aortic valve is located on the border between the left ventricle and the ascending aorta and regulates blood flow from the ventricle to the aorta during systole [75]. During the diastole, the backward pressure of the blood closes the valve and impedes the retrograde flow [75]. Macro- and micro- characteristics of aortic valve provide optimal functionality during the cardiac cycle.

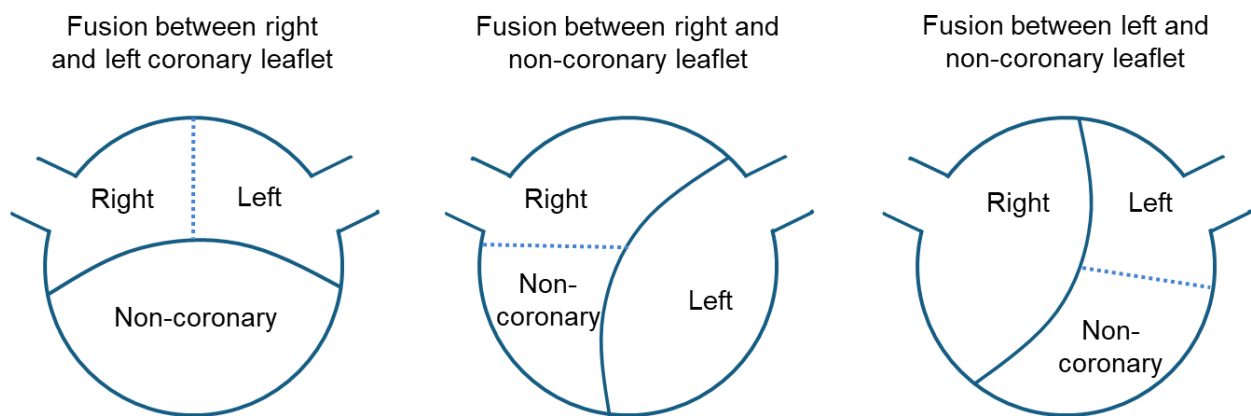
#### 1.1.1 Macrostructure of aortic valve

The aortic valve is constituted of three leaflets, left coronary, right coronary and noncoronary leaflets, which are named in accordance with location of coronary artery ostia (Figure 1) [76]. Each leaflet is attached to the fibrous annulus at one edge and free at the other [77]. In the closed position, free edges form a coaptation zone extending from the commissures to the center of the valve [75,76]. In the middle of each free edge there is a thickened fibrous bulge, noduli of Arantii, which promote more tight connection of cusps during the diastole [76]. Immediately beyond to the aortic valve, an aortic anatomical dilation is observed, together with the leaflets this widening forms three aortic sinuses [76,78].



**Figure 1. Macrostructure of aortic valve shown as a cut between the right and left coronary leaflets (adapted from reference [76])**

Of note, 1-2% of population have congenitally malformed bicuspid aortic valves [79]. The bicuspid aortic valve consists of two leaflets, which often have unequal size (Figure 2). The larger one is mostly formed by fusion of the right and the non-coronary leaflets or right and left coronary leaflets; left and non-coronary leaflets are observed very rarely [80], as result of incomplete separation of the valve during embryonic development [81]

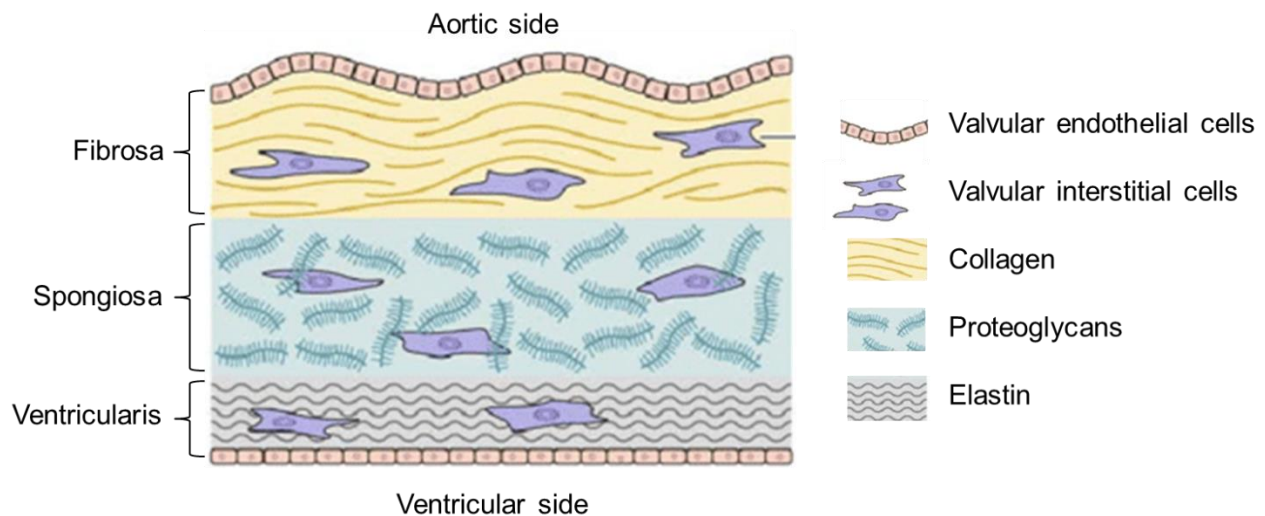


**Figure 2. Schematic illustration of the morphology variations of bicuspid aortic valves**

## 1.1.2 Microstructure of aortic valve

### 1.1.2.1 Organization

The unique biomechanical properties of aortic leaflets provide them with the ability to resist structural deformation with each heartbeat for decades. This is achieved by its cellular composition and micro organization of the extracellular matrix (ECM) layers: fibrosa, spongiosa, and ventricularis (Figure 3) [1]. The fibrosa is situated on the aortic side of the valve. It consists of circumferentially oriented type I and III collagen fibres and is the main load-carrying layer [82,83]. On the ventricular side of leaflets is the ventricularis layer, which consists of collagen and radially arranged elastin fibres with cross-links [82,83]. These two layers are separated by a layer of proteoglycans, scattered collagen and elastin fibers, called spongiosa [1,75,82,83]. Its main function is compressibility and facilitation of shearing between fibrosa and ventricularis during the heartbeat [1,75,82].



**Figure 3. Three layer organization of extracellular matrix and cellular composition of aortic valve leaflets (modified from reference [84])**

### **1.1.2.2 Cell types**

Aortic valves cells are mainly represented by valvular endothelial cells (VECs) and valvular interstitial cells (VICs) [85,86]. A low number of immune cells and smooth muscle cells might also be found scattered throughout healthy valve tissue [28,87,88].

VECs create a monolayer on the blood-connecting surface of the leaflet. In response to shear stress they align perpendicular to flow and form a physical barrier between the blood and valve tissue [89,90]. VECs are characterized by expression of endothelial specific markers such as CD31+ and von Willebrand factor [91]. VECs control adhesion of inflammatory cells on leaflets [92] and transfer of molecules from the blood [93]. Interestingly, the mechanical properties and phenotypic profile of VECs are side-specific and might be explained by different hemodynamic forces applied to ventricular and aortic endothelial layers [27,94–96].

The most abundant cells in the aortic valve are the VICs. They are represented in all three layers of the leaflets. In healthy valves, VICs primary function is to maintain valve integrity, which includes degradation of the ECM by matrix metalloproteinases (MMPs) [97] and secretion of new collagen fibers [98]. The most commonly used markers for VICs are alpha smooth muscle actin ( $\alpha$ SMA), smooth muscle myosin, vimentin, and desmin [85]. Depending on functional differences, Liu and colleagues distinguished five phenotypes of VICs: embryonic progenitor endothelial/mesenchymal cells, quiescent VICs, activated VICs, progenitor VICs, and osteoblastic VICs [99]. Activated VICs, progenitor VICs, and osteoblastic VICs are suggested to populate only calcified valves [99]. Interestingly, VICs isolated from healthy and calcified valves may have different expression levels of the same cell specific markers [85]. For instance, VICs isolated from calcified valves are characterized by a higher expression of  $\alpha$ SMA and desmin than cells from healthy valves [85]. In addition, VICs obtained from stenotic valves possess a reduced differentiation potential in comparison with VICs from healthy valves [100].

The interaction between VICs and VECs is very important for maintaining valve homeostasis. Disturbance of balanced crosstalk between VECs and VICs might lead to AS development. Co-culture of porcine cells revealed that VECs might regulate the VIC phenotype toward quiescent by decreasing their  $\alpha$ SMA expression [90]. In support of this crosstalk, a study on VIC-VEC interactions showed that VECs inhibit VICs calcification by downregulation of the expression of runt related transcription factor 2 (Runx2),  $\alpha$ SMA, and osteocalcin [95].

## 1.2 Aortic stenosis

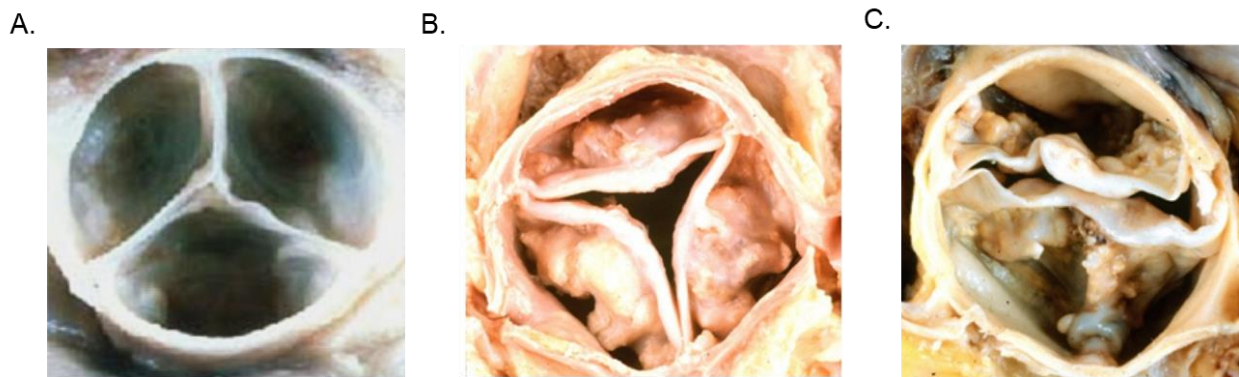
AS is a disease characterized by narrowing and obstruction of the aortic valve opening due to fibro-calcific remodeling and thickening of aortic valve leaflets, which impairs blood outflow from the left ventricle and induces pathological changes in it [1]. The diagnosis and severity (mild, moderate and severe) of AS is mostly defined based on echocardiographic evaluation of haemodynamic parameters, such as peak aortic jet velocity, mean gradient and aortic valve area [101] (Table 1). Patients with mild and moderate AS are asymptomatic, while patients with severe AS may or may not exhibit symptoms, which include shortness of breath, chest pain, fainting and heart failure [101].

**Table 1. Hemodynamic classification of aortic stenosis severity (adapted from reference [1])**

Parameter	Aortic sclerosis	Mild AS	Moderate AS	Severe AS
Peak aortic jet velocity	<2 m/s	2-3 m/s	3-4 m/s	$\geq$ 4 m/s
Mean gradient	<10 mm Hg	10-19 mm Hg	20-39 mmHg	$\geq$ 40 mm Hg
Aortic valve area	>2.0 cm <sup>2</sup>	1.6 – 2.0 cm <sup>2</sup>	1.1 – 1.5 cm <sup>2</sup>	$\leq$ 1.0 cm <sup>2</sup>

Development of AS is considered as an actively-regulated process, which shares a lot of similarities in its development with atherogenesis, such as lipid accumulation, inflammation and calcification [1]. The clinical manifestation of AS is preceded by aortic valve sclerosis [102]. The latter is distinguished from AS by the absence of valve obstruction [1]. In rare cases, AS can be triggered by non-sterile inflammation, in particular by rheumatic fever [103], or diagnosed as non-calcific AS. However, one of the main aetiologies of AS is calcified aortic valve disease (CAVD) [104,105], characterized by progressive leaflet calcification [104,105] (Figure 4).

Currently, there are no effective drug therapies which can moderate its progression. The only established treatments for AS are transcatheter or surgical valve replacement. Thus, better understanding of the mechanisms of AS development for prevention and treatment are required.



**Figure 4. Healthy tricuspid aortic valve (A), stenotic tricuspid aortic valve (B) and stenotic bicuspid aortic valve (C) (modified from reference [106])**

### 1.3 Epidemiology of AS

AS is the most common valvulopathy in Europe. The prevalence of AS, in both patients with bicuspid and tricuspid valves, increases markedly with age [11]. In the general population, aortic valve calcification is observed in 13% of people over 45 years of age [107], while in people over 60 years of age it reaches 33% [108]. In the Cardiovascular Health Study,

which enrolled subjects older than 65 years, 26% of the healthy cohort had aortic sclerosis and 2% had AS [3]. In the Tromso study, AS was detected in 0.2% of 50-59 year old citizens and up to 10% in those over 80 years [2].

The disease might progress quite fast and it takes only 5 years for approximately 9% of patients with an aortic valve sclerosis to develop AS [109]. Another study, which enrolled patients with aortic valve sclerosis, showed that within 7 years AS developed in 16% of all participants [102]. Notably, the mortality rate in untreated patients with severe AS and cardiac symptoms is estimated to be up to 50% within 1 year [12]. Furthermore, in connection with the growing elderly population, prevalence of AS has been predicted to increase in future years [4].

#### **1.4 Risk factors**

Advanced age, male sex and elevated levels of plasma lipoprotein (a) (Lp(a)) are the most serious risk factors in AS development [3]. Among others, hypertension, smoking, obesity, diabetes mellitus and chronic kidney disease were reported [110]. A lot of these risk factors are also common for atherosclerosis, and often patients with AS have concomitant atherosclerotic lesions in coronary arteries [111].

All AS risk factors can be divided into two groups: non-modifiable and modifiable. Non-modifiable factors include age, sex and genetic predisposition (family history), while all other risk factors mentioned above belong to modifiable factors and lifestyle changes may reduce AS incidence [112]. For instance, smoking is considered to be dose-dependent risk factor and smoking cessation leads to a time-dependent decrease in AS risk [7]. Thus, better understanding of effectiveness of risk modifications could give a potential insight on the design of preventive measures [110].

An increasing number of studies have found that genetics might be implicated in AS development [113–116]. Genetic predisposition to AS was found in patients, who carry the B

allele of vitamin D receptor [117]. It was assumed that this allele might alter calcium absorption and lead to an increase in bone loss [117]. Also, the genetic variation rs10455872 in the *LPA*, gene that encodes apolipoprotein(a), was reported to double the risk of aortic valve calcification via elevated plasma Lp(a) and was also associated with incident AS [115]. Likewise, elevated plasma levels of Lp(a) and oxidized phospholipids were associated with increased valvular calcification, faster disease progression and an increased risk of cardiovascular events [118]. In addition, a meta-analysis of two genome-wide association studies (GWASs) has revealed AS-associated variants of *RUNX2* and *CACNA1C* genes [116], which encode transcription factors involved in osteoblast differentiation and subunits of a voltage-dependent calcium channel belonging to the calcium signaling pathway respectively.

#### **1.4.1 Bicuspid aortic valves as risk of AS**

AS occurs with approximately the same prevalence in the anatomically normal tricuspid valve and the abnormal bicuspid valve [11]. The atypical anatomy of bicuspid valves leads to unsteady shear stresses under physiological flow and can play a role in accelerated valve calcification [27,119]. Thus, individuals with a bicuspid aortic valve develop the disease at a younger age comparing with those with tricuspid valves [11,120].

Bicuspid valve malformation could be inherited and its heritability is estimated to be up to 89% [121]. Currently, there are no single robust models that explain the inheritance of bicuspid valves. However, recently discovered mutations in several genes might partly shed some light on this issue. For instance, Garg et al. described mutations in gene *NOTCH1* associated with bicuspid aortic valves in several generations of two families [122]. Another missense mutation in *NOTCH1*, revealed by Mohamed and coworkers, explains 4% of sporadic bicuspid valve cases [123]. *NOTCH1* encodes a transmembrane receptor involved in signaling, which controls cell fate and developmental processes [124]. Interestingly, an *in*

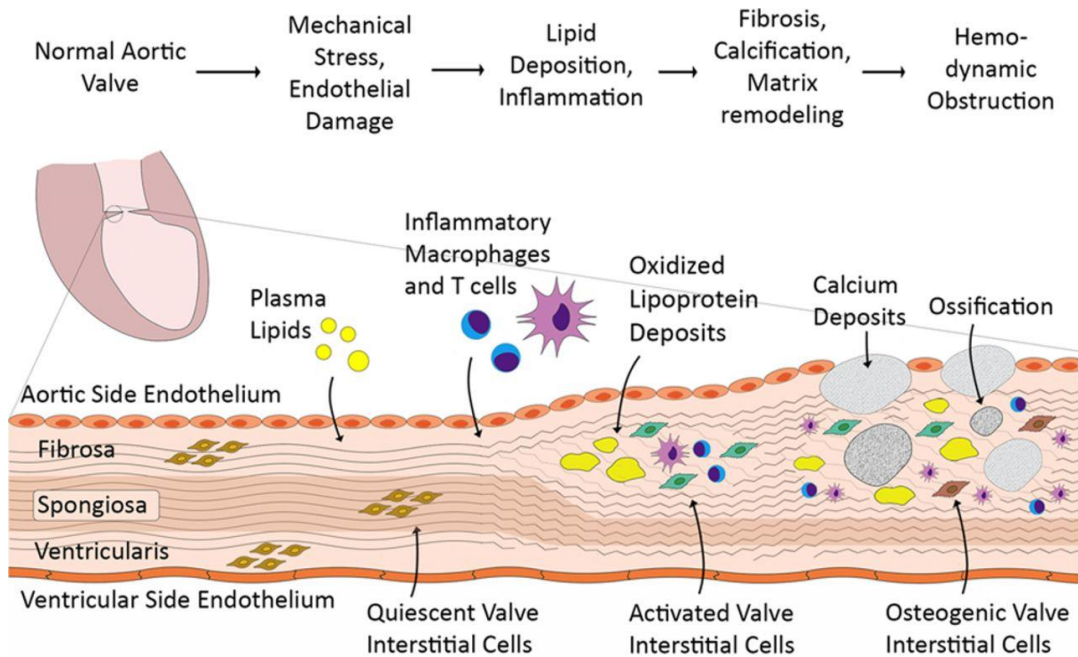


*vitro* study showed that early Notch-dependent mechanisms of calcification are different in VICs of bicuspid and tricuspid aortic valves [125].

One more polymorphism associated with approximately 2% of sporadic cases of bicuspid aortic valve in humans was found in *GATA5* [126], a gene that encodes a transcription factor, which coordinates heart and valve development [127]. Knockout of *Gata5* in mice leads to the development of bicuspid aortic valve by fusion of the right-coronary and noncoronary leaflets [128]. Recently, using next-generation sequencing, a large number of novel genetic variants have been found to be associated with bicuspid aortic valve [129,130], further studies are needed for a more precise estimation.

## **1.5 Aortic valve calcification**

Aortic valve calcification development is an active process of lipid accumulation [131], inflammatory cell infiltration [28,32], extracellular matrix remodelling [82], VICs transdifferentiation [85], cell death [82] and calcific mineral deposition [28] (Figure 5). According to *in vivo* molecular imaging techniques CAVD development could be divided into three phases [132]. The first (initiation) phase is characterized by lipid deposition, infiltration of immune cells and VIC activation [46,132,133]. These processes are preceded by endothelial denudation due to local haemodynamic stress and impaired wound healing [27,69]. Calcification starts mostly on the aortic side at the attachment of the valve to the aortic wall and propagates towards the tip [28,134]. The second (propagation) phase is accompanied by chronic inflammation, changes in extracellular matrix, pathological transdifferentiation of VICs and microcalcification [46,132,133]. The last phase, known as the late stage, is associated with progressive calcific mineral deposition [46,132,133].



**Figure 5. Pathogenesis of aortic valve calcification (from reference [135])**

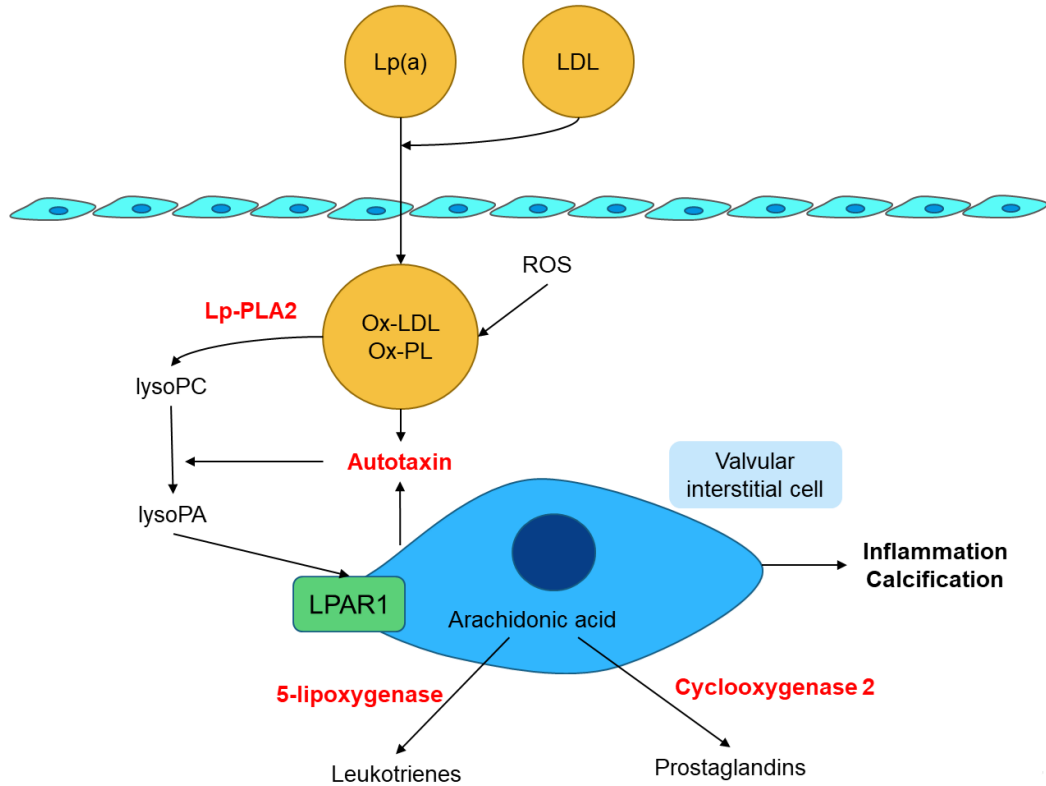
## 1.6 Lipids and lipid-derived mediators

Circulating lipoproteins might infiltrate into the fibrosa layer through a disrupted endothelial layer [131]. Accumulation of the apolipoproteins B, (a) and E in leaflets is associated with lipids deposition in aortic valve lesions since apolipoproteins bind lipids to form lipoproteins [131]. In valves, infiltrated lipids are retained by components of the extracellular matrix, in particular, biglycan, which is overexpressed in stenotic valves [136] and glycosaminoglycan, which was shown to be elongated in aortic valves under inflammatory conditions [137]. *In vitro* studies showed that Lp(a) induces calcification and osteogenic differentiation of VICs [138].

Oxidized phospholipids (OxPLs) have been shown to colocalize with Lp(a) [138]. There are two possible sources of OxPL in valves. First, they might be a product of free radical lipid oxidation during oxidative stress [139]. Second, they might infiltrate together with Lp(a) from plasma; it is known that 85% of the total amount of OxPL found in plasma is carried by Lp(a) [140]. Interestingly, elevated plasma levels of Lp(a) and OxPLs are associated with

increased valvular calcification activity, faster disease progression and an increased risk of cardiovascular events [118]. In aortic valves OxPLs activate signaling pathways that cause macrophages to undergo foam cell formation [141]. In addition, OxPLs induce inflammation, calcification and osteogenic differentiation of VICs *in vitro* [118,138,142].

Some lipid-derived mediators such as lysophosphatidic acid and eicosanoids are implicated also in the progression of aortic valve calcification [143–147]. Lipids accumulated in valve tissue might undergo enzymatic transformation by lipoprotein-associated phospholipase A2 (Lp-PLA2) and autotaxin, which are carried by lipoproteins and are overexpressed in human stenotic valves [143,144] (Figure 6). Lp-PLA2 hydrolyzes glycerophospholipids and produces free fatty acids and lysophosphatidylcholine. In turn, autotaxin uses lysophosphatidylcholine as a substrate to generate lysophosphatidic acid. Both, lysophosphatidylcholine and lysophosphatidic acid have been shown to possess proinflammatory properties and induce mineralization of aortic valves [143–145,148]. Nagy and colleagues demonstrated that 5-lipoxygenase, an enzyme, which synthesizes leukotrienes, is upregulated in stenotic valves [19]. Administration of leukotriene C4 in valvular myofibroblast cultures induced osteogenic pathways and calcium nodule formation [19]. In a subsequent study, it was shown that VICs can transform into immune-like cells and enhance inflammation through increased 5-lipoxygenase expression [146]. Finally, increased expression of prostaglandin-synthesizing enzyme cyclooxygenase 2 have been shown in stenotic valves [147]. Inhibition of this synthase in VICs led to reduced osteogenic gene expression and cell calcification [147].



**Figure 6. Metabolism of lipids in aortic valve (modified from reference [1])**

Lp-PLA2 = lipoprotein-associated phospholipase A2; LPAR1 = lysophosphatidic acid receptor 1, LDL = low-density lipoprotein LDL, PL = phospholipid

## 1.7 Oxidative stress

Oxidative stress contributes to the development of AS. Indeed, calcified aortic valve lesions are characterized by elevated production of reactive oxygen species (ROS), which are the result of increased expression of nicotinamide adenine dinucleotide phosphate oxidase [33], uncoupled nitric oxide synthase function [34], decreased antioxidant capacity [34,36] and iron accumulation [149,150]. Oxidative stress has multiple effects on biological systems. As mentioned before, ROS has an impact on aortic valve calcification through oxidation of lipids. Secondly, ROS can cause epigenetic changes in cells. For instance, oxidation of the guanine in *TNF- $\alpha$*  gene promoter regions can enhance their transcription via the base excision repair pathway [151]. In addition, oxidative stress may be implicated in

atherogenesis through changes in the methylation pattern of the DNA (demethylation or hypermethylation) [152]. Immunohistochemical staining of calcified aortic valves revealed that oxidative stress is associated with DNA damage and accumulation of 8-oxoG in the lesions [36]. Finally, it has been demonstrated *in vitro* that oxidative stress can drive VICs transdifferentiation via the AKT signaling pathway [36].

## 1.8 VICs differentiation

VICs are very plastic cells and their phenotype evolves throughout life in response to environmental changes [153]. *In utero* VICs have a myofibroblast-like phenotype, while from birth to adulthood they undergo phenotypic modulation and become quiescent fibroblast-like cells [153]. Isolated VICs are a heterogeneous population of cells that may exhibit various phenotypic features. Treatment of these cells with different cell culture mediums may contribute to osteogenic, adipogenic, chondrogenic, and myofibroblast lineage transdifferentiation [154]. It is considered that the key cellular mechanisms that lead to aortic valve calcification are VICs differentiation into myofibroblast- and osteoblast-like cells [85]. Indeed, aortic valve calcification is often associated with osteogenic differentiation of VICs [155,156]. A lot of bone matrix and osteoblast markers such as osteopontin, bone sialoprotein, osteocalcin and the osteoblast-specific transcription factor Cbfa1 were found to be upregulated in calcified aortic valves [156]. Moreover, histopathological studies revealed that 13% of human calcified aortic valves are characterized with lamellar bone, which consists of osteoblasts and osteoclasts [157], while myofibroblast-like cells are also widespread in diseased aortic valves [158].

Activation and further transdifferentiation of VICs to osteoblast-like and myofibroblast-like cells can be triggered by altered mechanical stress and biochemical stimuli [85]. Of note, myofibroblasts are characterized by their ability to contract the extracellular matrix, which is provided by high levels of  $\alpha$ SMA expression, while osteogenic differentiation is associated with increased alkaline phosphatase (ALP) activity and expression of osteogenic mediators,

such as bone morphogenetic protein 2 (BMP2) and Runx2 [85]. In cell culture, VICs are inducible toward myofibroblast phenotype by transforming growth factor beta (TGF- $\beta$ ) stimuli and by a stiff matrix [85,99,159,160]. In turn, VICs may also differentiate into osteogenic-like cells under the influence of inflammation and mechanical stress [161], as well as by stimulation with BMP2 [162]. The latter induces expression of Runx2 [162], which is the key transcriptional regulator of the osteoblastic lineage. Transdifferentiated VICs contribute to the calcification of the aortic valve through: i) expression of cytokines, which stimulate inflammation and further transdifferentiation of the VIC, ii) production of ALP, which can directly contribute to the mineralization of the valve and finally, iii) apoptosis, as it is known that cell debris serve as nucleation sites during calcification [85].

## **1.9 Immune cells infiltration**

Healthy valves include a small number of immune cells, which are mainly represented by scattered macrophages and mast cells, while lymphocytes are mostly not detected [28,87,163]. However, during disease development inflammatory cells infiltrate abundantly aortic leaflets [32,133]. This process is mediated by elevated expression of endothelial adhesion molecules, such as intercellular adhesion molecule 1, vascular cell adhesion molecule 1 and endothelial selectin (E-selectin) [92,164,165]. Calcific aortic valves present chronic inflammatory features with infiltration of both innate and adaptive immunity cells [32,87,166].

In aortic valves, innate immune cells are mostly represented by macrophages [28] and mast cells [87]. During AS development macrophages infiltrate abundantly aortic leaflets and form aggregates, which can also contain lipid-laden foam cells [28]. Macrophages are predominantly represented by a M1 subtype characterized by secretion of high levels of pro-inflammatory cytokines [167]. Mast cells are found to be scattered throughout the stenotic valve with higher density near the calcified areas [163], and are thought to be associated with lamellar bone in calcified valves [157]. These innate immune cells secrete cytokines,

such as tumor necrosis factor alpha (TNF- $\alpha$ ), which induce expression of interleukin-1 beta (IL-1 $\beta$ ) and interleukin-6 (IL-6) through activation of the nuclear factor kappa B (NF- $\kappa$ B) pathway [1]. In VICs IL-1 $\beta$  promotes expression of MMPs, which degrade the matrix and contribute to neo-vascularization [168]. In turn, administration of IL-6 and TNF- $\alpha$  in VICs induce expression of BMP2 and receptor activation of the nuclear factor kappa-B ligand, which in turn stimulate osteogenic transdifferentiation and mineralization of VICs [169–171]. In addition, innate immune cells are source of proteases, such as MMPs and cathepsins, which causes degradation of elastin and collagen [172–175] and thus directly contribute to ECM remodeling.

Currently, the knowledge about adaptive immunity cell implication in aortic valve stenosis is very limited. A adaptive immune cell present in stenotic valves is represented by T and B cells [87]. They are present in lesions surrounding calcified nodules and areas of neo-vascularization, and have a general distribution [28,63,157,176,177]. Since T cells in aortic valves consist of expanded clones [63], it suggests possible implication of an antigen-specific immune response in CAVD development. However, epitopes that may activate lymphocytes, as well as implication of adaptive immunity in the CAVD pathogenesis response remains elusive [166].

### **1.10 Matrix remodelling**

Composition and organization of aortic valve extracellular matrix changes during development and aging [178,179]. However, significant alterations occur in the valvular ECM during AS establishment. Remodelling of ECM during CAVD includes degradation and synthesis of ECM components leading to changes in the three-layer organization, thickening of leaflets and loss of elasticity [82]. These alterations substantially affect mechanical properties of the valve [82].

Secretion of proteases such as MMPs and cathepsins play a key role in degradation of ECM components in valves [97,168,172,174,175,180–182]. In valves, these proteins are secreted by inflammatory cells, VICs and VECs [172,182,183]. Due to their enzymatic activity, these proteases are able to break down collagen and elastin fibers [82] as well as to cleave cell adhesion molecules and activate or deactivate signaling molecules [183]. Activity of MMPs is regulated by endogenous tissue inhibitors of metalloproteinases (TIMPs). Currently, there are many studies that have demonstrated the presence of TIMPs in aortic valves [97,175,180,181], however, results are divergent. In stenotic valves, expression of TIMPs was shown to be significantly increased, slightly different or unchanged in comparison with normal valves [183]. However, it is an imbalance in the MMP/TIMP ratio that leads to ECM remodeling.

Neo-synthesis of new collagen fibres was detected in stenotic valves [184]. Despite this phenomenon, the fraction of collagen in the total protein is at least twice as low in calcified valves than in healthy ones. It is estimated at 40% for the non-calcified regions and 10% in the calcified regions of stenotic valves [184]. It has been suggested that this collagen synthesis may be mediated by angiotensin II through activation of TGF- $\beta$ 1 expression [185]. Angiotensin II might be locally activated in calcified valves by chymase and angiotensin-converting enzyme or by cathepsin G, which are all expressed in stenotic valves [163,172]. In addition, O'Brien et al. reported that angiotensin-converting enzyme is likely to be delivered in aortic valve from blood with low-density lipoproteins [186].

AS progression is also associated with increased content and changes in fine structure of proteoglycans and glycosaminoglycans [136,137,187,188], which are synthesized by VICs in response to pathological stimuli [137,188]. Proteoglycans and glycosaminoglycans are abundant in regions of calcified nodule formation in calcified aortic valves [187]. It is suggested that they contribute to disease development by lipid retention [136] and sequestration of mediators, which further facilitate their availability for cells [189]. Proteoglycans, in particular biglycan, can activate extracellular signal-regulated kinase 1/2



[190] and NF- $\kappa$ B [136] pathways through toll-like receptors, thus accelerating the inflammation process. Another proteoglycan, proteoglycan 4, which is upregulated in thickened and calcified regions of aortic valves, is known to promote transdifferentiation and calcification of VICs [188].

### **1.11 Neovascularization and intraleaflet haemorrhage**

Under normal conditions aortic valve structure displays no blood vessels [191]. However, neo-vascularization was observed in stenotic valves [28]. A study carried on 55 aortic valves from patients with nonrheumatic AS revealed neo-vascularization at different grades of classification with most prominent vascular density in moderate AS [191].

Neovascularization is tightly associated with chronic inflammation and tissue remodeling [32]. The thickening of the valve due to abnormal changes in ECM prevents normal oxygen diffusion into the leaflets and leads to hypoxia [191]. The insufficient oxygen supply induces expression of the hypoxia inducible factor alpha (HIF-1 $\alpha$ ) [192]. HIF-1 $\alpha$  is a transcriptional factor that promotes formation of blood vessels throughout induction of expression of vascular endothelial growth factor (VEGF) and endothelial nitric oxide synthase (eNOS) [193], central regulators of angiogenesis. In support of this involvement, immunohistochemical studies revealed upregulation of HIF-1 $\alpha$ , VEGF and eNOS in calcified aortic valves [191,192]. Another histopathological study performed on human stenotic aortic valves observed clusters and cord-like structures of CD34+ endothelial progenitor cells, which participate in angiogenesis [194]. Further studies are required to investigate role of progenitors in the calcified aortic valve neo-vascularization.

Neovascularization is associated with intraleaflet haemorrhage, which is observed in approximately 80% of human stenotic valves [195,196]. Areas of internal bleeding were detected in regions adjacent to neovascularization and associated with rapid progression of AS [195,196]. It was shown that intraleaflet haemorrhaging contributed to iron accumulation

by VICs in stenotic valves [149]. This leads to extracellular matrix remodeling, VICs proliferation and calcification [149]. In addition, iron may induce vascular damage through increased oxidative stress [150]. More recent evidence reveals that iron accumulation may precede calcification of aortic leaflets and even trigger it [69].

## **1.12 Biomineralization**

Valve mineralization is an active process of calcium phosphate complex deposition in aortic leaflets. Biomineralization is initiated and further facilitated by pathological changes in valve tissue. It continues gradually and leads eventually to massive calcium deposition. In the same stenotic valve calcific deposits can be observed as areas of heavily and lightly mineralized tissue, which correspond to different stages of the calcification process [197].

Ultrastructural analysis of calcific aortic valves revealed that calcium deposits are composed of various inorganic phases of calcium phosphate with different shapes and sizes [197–199]. Although 13% of calcified aortic valves are characterized with the presence of lamellar bone [157] the chemical composition of the calcium deposits is very similar to bone consisting of calcium, phosphorus, a carbonate group and small amounts of magnesium [197,198]. Mineralization in valves is different in comparison with bone formation. Besides spherical particles, which have a highly crystalline structure [198], mineralization of valves has dystrophic features and is mostly inhomogeneous and amorphous [200]. In addition, calcium deposits in aortic valves can contain small cavities with incorporated fibers of disorganized and mineralized collagen, which is the result of ECM remodeling [197].

It has been suggested that nucleation sites for calcium deposits arise on extracellular vesicles released by macrophages and VICs that undergo osteogenic reprogramming, as well as apoptotic bodies [1,201–203]. Apoptotic debris function as foci for dystrophic calcification that results in amorphous calcific deposits, whereas mineralization of secreted vesicles progress into well-organized particles [1]. Secreted vesicles are enriched with annexins and

tissue non-specific alkaline phosphate, which promote mineralization [201,203,204]. Both proteins have an impact on hydroxyapatite formation: annexins may facilitate harbouring of calcium in vesicles by acting as voltage-gated  $\text{Ca}^{2+}$  ion channels [201,203,205], while tissue non-specific alkaline phosphatase produce free phosphate by hydrolyze of inorganic pyrophosphate (PPi) [206]. Finally, extracellular vesicles are able to accumulate, to aggregate, to mineralize and to form microcalcifications [201,204]. Over time, microcalcifications progress into macrocalcifications [201].

### **1.13 Natural inhibitors of calcification and their imbalance in AS**

Healthy valves possess mechanisms that prevent their ectopic calcification. For instance, matrix Gla protein (MGP) is an ectopic inhibitor of calcification in aortic valves [207]. It has been shown to be overexpressed in the thickened areas of stenotic valves [47]. MGP acts in several different ways to prevent calcification. First, it can directly interact with hydroxyapatite crystals [207] and avert their accumulation in valves. Secondly, MGP directly binds to BMP2 [208] and possibly prevents its interaction with the BMP2 receptor. In addition, patients with aortic valve calcification have increased levels of the inactive forms of circulating MGP [209].

MGP can exist in two forms: an active form or an inactive form [210]. For its activation, MGP undergoes biochemical changes, which includes carboxylation or/and phosphorylation [210]. It is suggested that uncarboxylated and dephosphorylated MGP is fully inactive, while other forms possess different biological activities [210]. Activation of MGP is a vitamin K-dependent process [210]. Therefore, vitamin K deficiency as well as treatment with warfarin, an antagonist of vitamin K, are associated with higher levels of inactive forms of MGP in plasma [209,211]. Conversely, vitamin K supplementation leads to decreased amounts of uncarboxylated MGP in patients from the examined group [212]. Hence, it was suggested that vitamin K intake might slow AS progression [212].

PPi prevents calcification by suppression of extracellular hydroxyapatite crystal formation [213]. PPi is formed by hydrolysis of ATP by ectonucleotide pyrophosphatase/pyrophosphorylase (ENPP1). A study performed on mice showed that deletion of ENPP1 caused vascular calcification [214]. In turn, overexpression of ENPP1, which was observed in calcified aortic valves, leads also to calcification [215]. Two mechanisms, which explain how overexpressed ENPP1 can induce calcification, were proposed. First, high levels of ENPP1 cause depletion of extracellular ATP, which prevents apoptosis of VICs through activation of phosphoinositide 3-kinase/Akt signaling, a major survival pathway [215]. Second, generated PPi can be hydrolyzed to Pi by alkaline phosphatase, which is also highly expressed during calcification [215]. Subsequently, Pi levels surpasses PPi production and induce calcification of VICs *in vivo* [215].

Thus, an imbalance in preventing and promoting mechanisms of calcification leads to initiation and progression of AS.

## 2 Telomere biology

### 2.1 Telomere structure

Telomeres are specialized nucleoprotein structures at the extreme ends of linear chromosomes, composed of tandem-repeated DNA hexamer sequences bound by specific proteins [216,217]. These structures allow chromosomes' ends to be distinguished from DNA double strand breaks. In humans and other mammals, telomere DNA is represented by several thousand TTAGGG repeat sequences [218]. Most of the lengths telomeric DNA is double-stranded and terminates with single-stranded G-rich overhang in 3'OH (Figure 7A). To sequester chromosome ends, telomeric DNA curls around itself and the 3'OH single-stranded overhang invades into the double-stranded telomeric sequence, forming the T-loop and the D-loop at the invasion site [217,219] (Figure 7B). Telomeres contain T-loop structures throughout the cell cycle, except during the S-phase where the T-loop is unwound [220] and telomeres can be replicated.

Telomeres are further shaped and maintained by a complex of six-proteins (TRF1, TRF2, POT1, TPP1, TIN2 and RAP1) known as shelterin. Three of these proteins (TRF1, TRF2 and POT1) recognize directly TTAGGG repeats and bind to telomeric DNA. TRF1 and TRF2 cover a double-stranded part of the telomeres, whereas POT1 cover the 3'OH single-stranded overhang. POT1 forms a complex with TRF1 and TRF2 through TPP1 and TIN2, which bridges both TRF1 and TRF2. Finally, RAP1 connects exclusively with TRF2 [217] (Figure 7C). This elegant structure provides architectural protection of the chromosome ends which safeguards the telomeres from exonucleolytic degradation, end-to-end fusion and inappropriate activation of DNA repair machinery [217].



that besides the intrinsic end-replication problem, other factors can also contribute to telomere attrition.

### 2.3 Telomerase

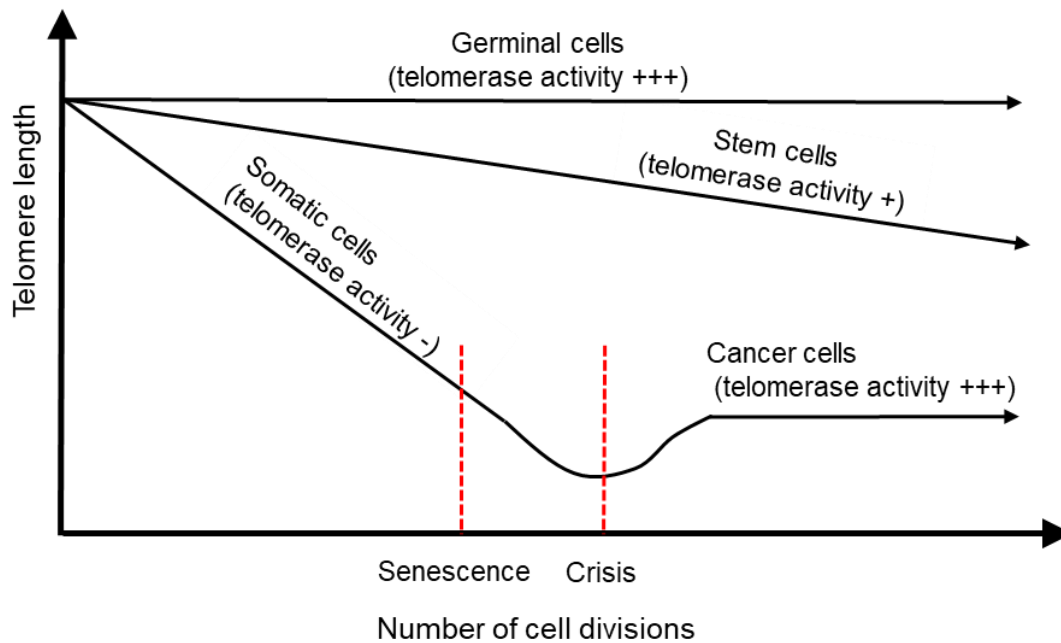
Some cells can preserve TL from attrition by expressing telomerase, an enzyme that adds telomeric repeats to the ends of chromosomes [227]. TL homeostasis in telomerase-positive cells is achieved by balancing the processes of attrition and replenishment, mainly due to telomerase activity.

Telomerase is a ribonucleoprotein complex. The core enzyme is composed of catalytic unit telomerase reverse transcriptase (TERT) and long non-coding RNA (TERC) that contains the template for telomeric repeat sequence synthesis [228]. The TERC component is expressed in all cells, whereas TERT is detected only in telomerase positive cells [229,230].

*In vitro* core enzymes can extend telomeric primers alone [231]. However, for *in vivo* activity, several accessory proteins are required for recruitment and binding of telomerase onto telomeres during the cell cycle [232]. The most crucial role is played by dyskerin, which stabilizes TERC [233] and by telomerase Cajal body protein 1, which accumulates telomerase in the Cajal body [234]. The last one is a non-membrane nuclear organelle, some functions of which are telomerase biogenesis and its trafficking to telomeres [235]

T-loop telomere structure makes G-overhang not accessible for telomerase in G1 and G2 phases of the cell cycle. During S-phase, the T-loop unfolds and the telomeric DNA becomes accessible to telomerase, which is recruited to telomeres in the late S-phase of the cell cycle and adds ~60nt to a single-strand telomere overhang [236]. Interestingly, short telomeres have a higher probability of being extended than long ones [237]. This might be due to inhibition of the telomerase activity by components of shelterin complexes and the load of shelterin proteins on telomeres may explain their length regulation [238].

In humans, telomerase activity is present in embryonic and germline tissues [239], it is also reactivated or up-regulated in 80-90% of tumors [240–243]. On the contrary, the vast majority of cells in adults lack telomerase activity [243] with the exception of stem cells [244] and activated lymphocytes [245], where levels of telomerase activity are low and insufficient to abrogate telomere shortening [243,246] (Figure 8).



**Figure 8. Telomere length dynamics in germinal, stem, somatic and cancer cells according to the presence of telomerase activity**

Interestingly, telomerase also displays telomere-unrelated functions known as non-canonical telomerase roles [247]. Indeed, comparison of the transcriptional profiles of normal and *hTERT*-immortalized fibroblasts revealed changes in 172 gene expression levels. These results suggested that telomerase activation may have a whole spectrum of biological consequences in addition to its function of telomere replenishment [248]. Subsequent studies in this field found that telomerase can localize in the mitochondria and sensitize cells to oxidative stress, which may lead to apoptotic cell death [249]. Moreover, telomerase was shown to upregulate expression of DNA methyltransferases [250,251] and regulate NF- $\kappa$ B



signaling pathways [252]. Likewise, it was found in animal models that telomerase, even with direct mutation in TERT catalytic subunit leading to abolishment of canonical telomerase activity, is involved in the regulation of Wnt signaling pathway. This regulation was done through physical interaction between TERT and BRG1, a SWI/SNF-related chromatin remodelling protein [253]. However, these findings were questioned in the independent study of Listerman *et al.* [254]. In this study overexpression of *hTERT* in cancer cell lines, which are known to have endogenous telomerase activity, did not confirm interaction of hTERT with BRG1, neither significant effect on the Wnt cascade. They suggest that hTERT do not modulate Wnt pathway universally but in a context-dependent way. However, another study found interactions between hTERT and BRG1 in Hela cancer cell line [255]. To sum up, taking into account the different contexts of those studies, as well as different methodological approaches and models, these results are not completely contradictory [247].

Besides telomerase containing full-length TERT subunit, which is catalytically active, those containing TERT splice variants and lacking catalytic activity are also involved in the regulation of different cellular processes [24]. Currently, 22 isoforms of human TERT (hTERT) have been described [25], however, the biological role of most of them has not yet been elucidated. Table 2 describes the biochemical functions of most studied hTERT alternative splice variants. It is interesting to note that some of these isoforms, so-called dominant-negative isoforms, were shown to inhibit telomerase activity of full-length telomerase. For instance, overexpressed minus beta [256], minus alpha [257], INS3 and INS4 [258] splice variants were suggested to inhibit canonical telomerase activity. However, besides minus beta *hTERT* isoform, which was proposed to compete with the catalytically active hTERT protein for *hTERC* [256], possible mechanisms of inhibition of telomerase activity remain unknown. In addition, it is worth to mention that another study using minus beta hTERT isoform didn't demonstrate dominant negative effect on telomerase activity [257]. This discrepancy might be explained by different levels of target protein expression.

**Table 2. Major spliced isoforms of human TERT (modified from reference [24])**

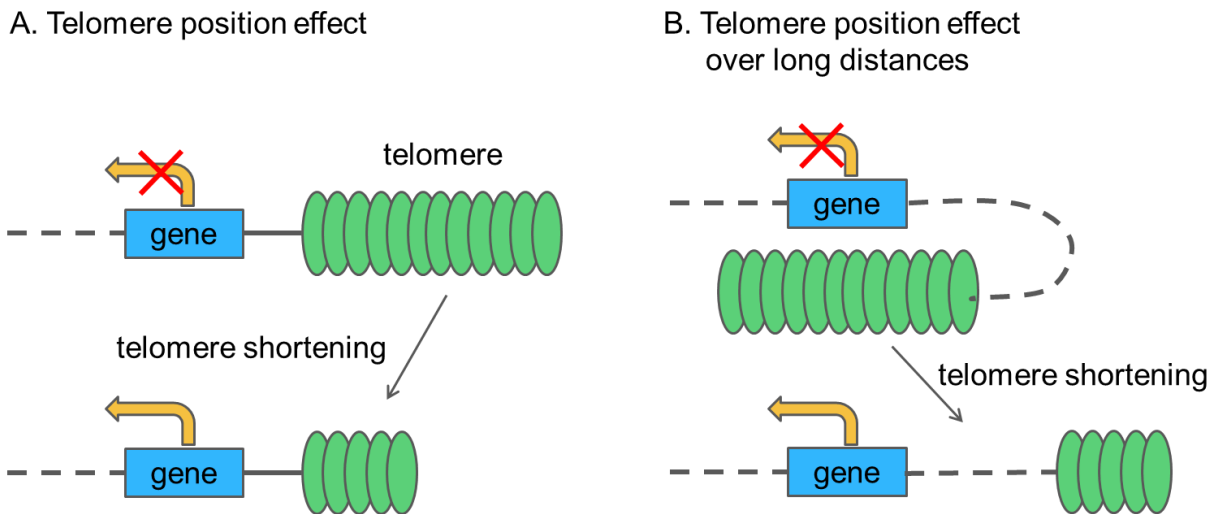
<b>Isoform</b>	<b>Biochemical Function</b>
<b>Full-length</b>	Functional hTERT protein, maintains telomeres when in active telomerase holoenzyme
<b>Minus beta</b>	Mostly degraded by non-sense mediated decay, some translated into protein and may play a role in DNA damage repair/ protection from apoptosis, may bind <i>hTERC</i>
<b>Minus alpha</b>	Dominant-negative, binds <i>hTERC</i>
<b>INS3</b>	Dominant-negative, binds <i>hTERC</i>
<b>INS4</b>	Dominant-negative, binds <i>hTERC</i>
<b>DEL2</b>	Proposed mitochondrial <i>hTERT</i> variant, retains <i>hTERT</i> mitochondria localization sequence in exon 1.
<b>Delta4–13</b>	Proposed to stimulate proliferation. Interacts with WNT/ $\beta$ -catenin.
<b>Minus Gamma</b>	Tissue specific and may inhibit telomerase action at the telomeres

#### **2.4 Implication of short telomeres in cellular processes**

Short telomeres induce a whole spectrum of changes in cells. First, the inability of short telomeres to maintain their structure leads to signalling that blocks the cell cycle, stopping cell division. This cell state is called replicative senescence [70]. Despite lacking proliferation activity, senescent cells remain viable and metabolically active [259]. They develop senescence-associated secretory phenotypes (SASPs) which are characterized by changes in protein expression patterns and increase secretion of inflammatory growth factors, cytokines, chemokines and MMPs [259,260]. These factors have paracrine activities promoting changes in tissue microenvironment and drive low-grade chronic inflammation, thus contributing to development of age-related diseases [259,260]. Moreover, senescent cells accumulate with age and may promote developing age-related diseases [259,261,262].

Conversely, targeted elimination of senescent cells, using genetic or chemical therapy, improves physical functions and prolongs healthspan [263–266].

Secondly, telomere attrition causes epigenetic changes in chromatin [267]. These changes are characterized by the loss of heterochromatic features in telomeric and subtelomeric regions due to decreased methylation and increased acetylation [267]. Such modifications are known to downregulate gene expression in close proximity to the telomere. This epigenetic phenomenon is called the telomere position effect (TPE) [51] (Figure 9A). TPE is limited and heterochromatin marks in subtelomeric regions do not extend more than 100 kb from the telomere [52,268]. Telomeres can also regulate gene expression via so-called TPE over long distances, a process that involves telomere looping and its interaction with upstream chromatin, which leads to silencing of the genes [16,53,54] (Figure 9B). Interestingly, the loop can be located at a distance up to 10 Mb from telomeres and the disruption between TPE-sensitive gene loci and long telomeres occurs upon telomere shortening to a critical length [16]. Taken together, these discoveries indicate that TL may play a role in cellular processes before replicative senescence.



**Figure 9. Telomere length dependent regulation of gene expression**

A. telomere position effect

B. telomere position effect over long distances (B) (adapted from reference [53]).

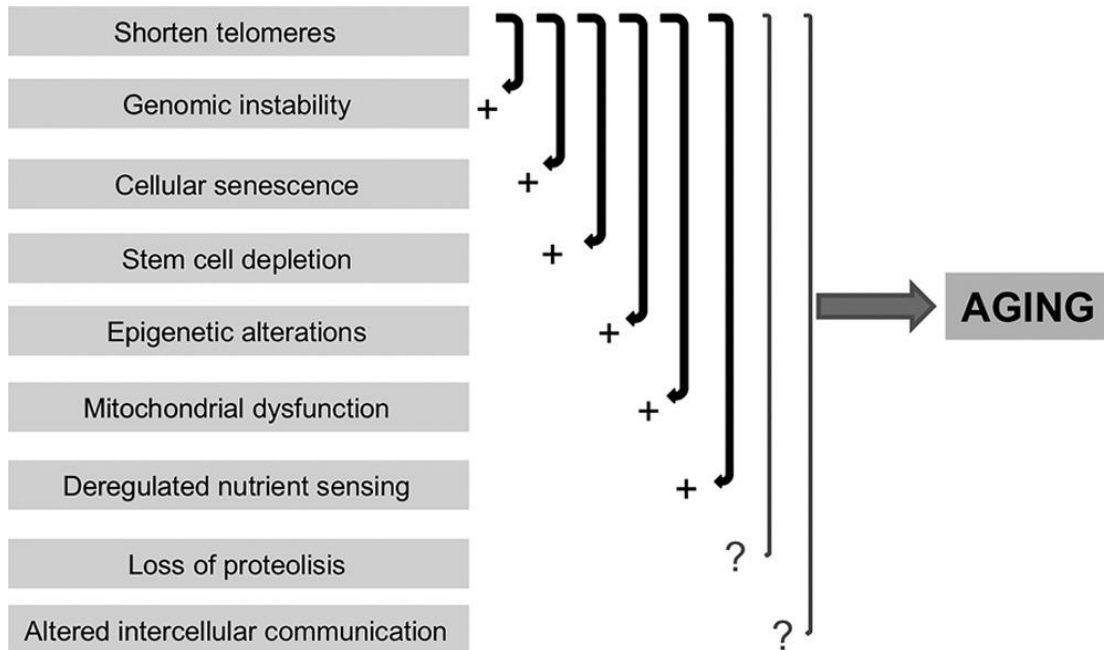
In addition, critically short telomeres are recognized as damaged DNA, which activates DNA damage response and as a result triggers growth arrest or apoptosis [70,269–271]. In some cases, the loss of tumor suppressors allows cells to bypass growth arrest and leads to a telomere crisis [271]. Cells in telomere crisis are characterized by genomic instability that can play a role in cancer development and progression [272].

## **2.5 Aging and telomeres**

Aging is a process accompanied by an accumulation of age-related changes, which over time lead to a progressive functional decline at the physiological level and, ultimately, death [273]. Current research on the biology of aging aims to better understand the cellular and molecular processes that underlie these changes as well as the processes that drives the onset of age-related diseases. In 2013, Lopez-Otin and colleagues described in their synthetic literature review nine hallmarks that contributed to aging phenotype. These hallmarks included: telomere attrition, genomic instability, epigenetic alterations, loss of proteostasis, cellular senescence, stem cell exhaustion, deregulated nutrient sensing, mitochondrial dysfunction, and altered intercellular communication [273]. Gaining knowledge on these processes helped to identify some of their common determinants. Recently, it was suggested that short telomeres may underline the majority of hallmarks of aging, namely genomic instability, epigenetic alterations, cellular senescence, stem cell exhaustion, deregulated nutrient sensing and mitochondrial dysfunction [14] (Figure 10). Therefore, the fact that short telomeres could be implicated in aging and age-related diseases explains much of the prevailing interest in telomere biology.

A study that focused on telomeres in humans showed that longer telomeres in people over 75 years were associated with more years of survival [274]. In another work, individuals over the age of 60 years, who had shorter leukocyte TL (LTL), demonstrated poorer survival and higher mortality rates from infectious and heart disease compared to those with longer LTL.

This supports the hypothesis that short telomeres in humans contributes to mortality in age-related diseases [275]. Thus, it is possible to assume that factors that determinate individual TL could also impose predisposition to develop age-related diseases and impact life expectancy.



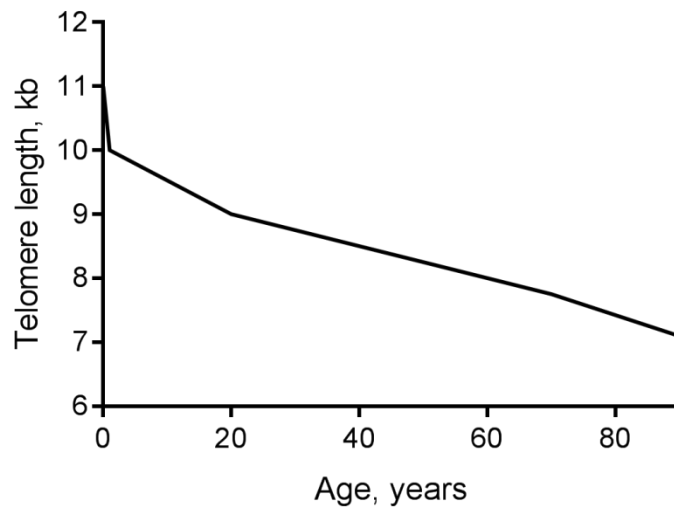
**Figure 10. Shorten telomeres induce biological processes involved in aging (from reference [14])**

## 2.6 Telomere dynamics in individuals and population

The mean TL at any point of the life course depends on the initial TL, telomere attrition and restoration by telomerase during the lifespan. Studies performed on tissues from newborns and fetuses have demonstrated that TL varies considerably between individuals at birth (4kb range), but display synchronicity in different types of tissue in one individual [57,58]. This synchrony may be accounted for by telomerase activity in somatic tissues during intra-uterine development [239]. This intra-individual variation holds in adult life, while tissues with high levels of proliferation represent shorter TL in comparison with minimally proliferative tissues [20,48,57,58]. The difference in TL between highly proliferative tissues (in particular

white blood cells, which reflect TL in hematopoietic stem cells) and minimally proliferative tissues (muscles) is explained by different levels of TL shortening in these tissues [20]. In adults telomere attrition in leukocytes is estimated at 25 base/year [276], while for somatic tissues like muscles mean telomere loss rates are of 15 base pairs per year [20]. Moreover, the gap in TL between leukocytes and muscle is already observed in early life and is accounted for by different proliferative history of these tissues [277]. Nevertheless, in adults, TL remains equivalent across somatic tissues within the individual [48].

Accumulating evidence from cross-sectional and longitudinal studies on LTL give us an insight into age-related telomere dynamics in populations. For instance, cross-sectional studies on leukocytes have shown that during the first years of life telomere attrition is very rapid and can exceed 1 kilobase per year [278,279]. Subsequent longitudinal studies on children suggest that the biggest decline in LTL appears before the second decade of life [280]. During the second decade of life, telomere attrition in leukocytes keeps at high rates and is twice as fast as during adulthood [280]. In adults, an average rate of LTL shortening remains at the same rate (25 base/year [276]) until the 7<sup>th</sup> decade of life and then it accelerates slightly [281] (Figure 11). A longitudinal study performed on 1156 participants that donated samples 12 years apart showed that LTL ranking was fixed in adults [282]. Basically, it means that people with relatively short LTL in comparison to their peers display short LTL during the lifespan. Conversely, individuals with relatively long telomeres preserve long telomeres in comparison to their peers throughout life course despite telomere attrition.



**Figure 11. Schematic representation of leukocyte telomere length decline in humans from birth to 90 years old**

## 2.7 Determinants of TL

Inter-individual variation of TL at birth currently is explained by several factors. They include inherited differences in TL, polymorphisms and mutations of genes involved in telomere maintenance, gender, paternal age at conception and race.

### 2.7.1 Influence of heritability on TL

Telomere length is a highly inherited trait [283,284]. This inheritance is driven by both genetic (see 2.7.2) and non-genetic mechanisms. It is suggested that non-genetic TL inheritance stems from direct TL transmission through parents' germ cells that participate in the formation of fertilized egg cell [285]. Further credence to this assumption comes from studies which have shown that TL of individual chromosome might be inherited [286,287]. Studies conducted on twins and families allowed to scale the influence of heritability on TL. According to examinations that do not segregate the influence of shared environment, heritability ranged between 56 and 84% [223,288–290]. When shared environment was

modelled in the study, heritability reached only 36% and shared family effect was estimated at 49% [291].

## **2.7.2 Genetic determinism of TL**

Genome-wide association studies (GWASs) have shown that inter-individual variations of TL can be regulated by several loci that contain genes involved in telomere biology [289,291–294]. Among these, *TERC* and *TERT* genes, which code for RNA and protein components of telomerase respectively were observed. Of note, mutations in *TERC* have been shown to be linked to short telomeres in leukocytes, but not in muscle [62]. Other genes such as *NAF1*, which is important for telomerase assembly [295], *RTEL1*, which is involved in replication and maintenance of telomeres [296,297], *OBFC1*, which regulates TL [298,299], *DDX11*, which encodes DNA helicase that maintains genome integrity [289] and *CTC1*, which plays a role in telomere maintenance [294] were reported to have single-nucleotide polymorphisms (SNPs) associated with LTL [289,291–294]. In addition 19p21 and 2p16.2 loci, without distinct gene candidates [292] and *BICD1* [293], *ZNF676* [294], *DCAF4* genes [300] whose role in telomere biology is unknown, were revealed to be linked to TL. It is worth noting, that the biggest meta-analysis of 14 GWASs, which determined seven loci with 57-117 base pairs effect per allele on LTL, explained less than 1% of its variance [292].

### **2.7.2.1 Telomeropathies**

Germline mutations in genes of proteins involved in telomere maintenance cause development of telomeropathies, a spectrum of disorders characterized by premature telomere shortening and premature aging [301]. Among genes identified to induce telomeropathies are genes that encode: components of the telomerase complex (*TERT*, *TERC*, *DCK1*, *NOP10*, *NHP2*), shelterin components (*TIN2*, *TPP1*), telomerase trafficking



(*TCBA1*), telomere synthesis (*RTEL1*, *CTC1*) and TERC RNA processing (*PARN*, *NAF1*) [302]. In humans, telomeropathies include such diseases as congenital dyskeratosis [233,303], aplastic anemia [304], pulmonary fibrosis [305,306], Hoyeraal-Hreidarsson syndrome [307], and liver disorders [308].

### **2.7.3 TL and gender effect**

Population studies have shown that, on average, women have approximately 200 bases longer LTL than men [309–311]. The first proposed explanation of this phenomenon was a slower rate of LTL attrition in women [312], which could be accounted for by potential estrogen-mediated stimulation of telomerase activity [313]. However, subsequent longitudinal studies in this field are controversial. On the one hand, Chen et al. published that mean rates of LTL shortening are modestly slower in young women than in men [314]. On the other hand, the Dalgard et al. study showed that premenopausal women have higher rates of LTL attrition compared to same age men, while after menopause opposite results were observed [315]. Recently, it has been suggested that the sex-related gap in LTL could be established before adulthood. This assumption was confirmed by detection of gender differences in LTL in newborns [316] and children at four years of age [317]. A study of twins, which showed that in non-identical twins, female LTL was equal to that of males, while identical twins LTL was longer in females than in males, also corroborate this hypothesis [318]. The absence of the gender difference in LTL of non-identical twins might be explained by masculinization of the female fetus under the influence of the brother's testosterone *in utero* [319].

### **2.7.4 Paternal age and TL**

Paternal age at conception can influence TL in offspring where findings showed that advanced paternal age is associated with longer telomeres in the children [317,320–325].

This presumably can be explained by longer TL in sperm of older individuals [324,326]. Two possible explanations were proposed, the first being that telomerase might slightly elongate telomeres in germline throughout lifespan [326]. As a second explanation, it was suggested that germ cells with short TL might be eliminated with advancing age due to selection pressure, resulting in emergence of a subset of sperm with longer telomeres in older men [324]. This assumption was reinforced by a study in monozygotic (MZ) and dizygotic (DZ) twins, in which DZ twins of older fathers displayed a decrease in the intra-pair variation in LTL, whereas in MZ twins these variation was stable regardless of paternal age. The authors used an earlier proposed hypothesis of age-dependent selection of germ cells to explain the observed decrease in the intra-pair variation in LTL of DZ twins of older fathers [320].

### **2.7.5 Ethnicity and TL**

Another factor that can influence TL is ethnicity/race [327–329]. Individuals from European ancestry typically have shorter telomeres than individuals from Africa [327,329,330]. Furthermore, recent research has shown that a difference exists even between African American ancestry and sub-Saharan African ancestry, where the latter group displays longer LTL [330]. The shorter LTL in Europeans than Africans might partially explain ethnic differences in risks of developing diseases that are associated with LTL, such as cancer and cardiovascular diseases [330]. Variations in TL were also observed in children with different ascribed ethnic identification. For instance, European children have shorter TL in saliva than Māori, Pacific and Asian children [317].

## **2.8 Effect of oxidative stress on TL shortening**

A vast amount of *in vivo* and *in vitro* studies showed that oxidative stress and chronic inflammation accelerate telomere shortening through telomeric DNA damage induced by

ROS [37–40,331–334]. ROS are able to generate about 100 DNA lesions [335], one of the most abundant is considered to be 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxoG) [336]. Given that DNA is enriched with guanine in telomeres [218], they are more vulnerable to oxidative stress damage by ROS than other sequences [42,43]. Furthermore, *in vitro* studies showed that telomeric sequence exposed to ROS contain 4.5 times more 8-oxoG [42] and up to 7 fold DNA breaks [43] than non-telomeric sequences with the same guanine ratio. In addition, repair of damaged telomeres are substantially delayed in comparison with bulk genome [337] or even are irreparable and trigger persistent DNA damage response and cellular senescence [338,339]. By using a tool that induced local oxidation of telomeres, recent study provided direct evidence that ROS can drive telomere attrition and telomere crisis (genome instability due to chromosome fusion) [38]. Besides DNA damage, oxidative stress has multiple effect on biological systems and thus telomere attrition could also be an aftereffect of improper telomere maintenance [31,37,302,340] caused by ROS.

A number of studies in mice indicate that oxidative stress may accelerate telomere attrition *in vivo* [39,341]. In humans, increased oxidative stress has been linked to some cardiovascular risk factors like obesity, smoking, diabetes and alcohol consumption [342,343]. It is well established that these cardiovascular risk factors are associated with shorter LTL in cross-sectional studies [344–347]. However, longitudinal observations have not confirmed these findings. For instance, examination of TL in 4576 individuals at an interval of 10 years did not reveal associations between telomere attrition and smoking or increased body weight [348]. Likewise, insulin resistance in patients with type 2 diabetes mellitus did not accelerate LTL attrition [349]. Similarly, a meta-analysis of 18 longitudinal cohorts confirmed shorter LTL in smokers than in non-smokers in a cross-sectional observation, whereas longitudinal LTL attrition rates were equal in smokers and non-smokers [350].

Further investigations are required to better understand the effects of oxidative stress on telomere attrition in humans.

## **2.9 Telomere length and diseases**

LTL is associated with a wide variety of illness, such as cancer, metabolic disease and age-related degenerative diseases. For instance, in prospective studies, long telomeres have been shown to be linked to lung cancer [351,352] and melanoma [353]. Likewise, a mendelian randomization study of 21 datasets revealed that genetic predisposition to longer LTL increase the risk for melanoma, lung adenocarcinoma, glioma, neuroblastoma and serous low-malignant-potential ovarian cancer, while genetic predisposition to shorter LTL increased the risk of cardiovascular disease [354]. In addition, short telomeres were associated with insulin resistance in subjects with type 2 diabetes mellitus [345,355], Alzheimer's diseases [356] and whole spectrum of cardiovascular diseases (discussed below). Despite the great interest in telomeres, the role of TL in the onset and development of the diseases has yet to be established.

## **2.10 Telomeres and cardiovascular diseases**

An increasing number of epidemiological studies, with some exceptions [357–359] have reported that cardiovascular diseases are associated with short LTL. For instance, short LTL was associated with heart failure [360], stroke [361] and degenerative AS [59], hypertension [362,363], ischemic heart disease [364], premature myocardial infarction [365], atherosclerosis [20,366,367] and cardiovascular mortality [368]. In addition, short telomeres are associated with cardiovascular risk factors such as obesity [346,369], insulin resistance [349,363], and a sedentary life style [370].

One of the most studied cardiovascular disorders in terms of telomere biology is atherosclerosis. Patients with atherosclerotic cardiovascular diseases (ASCVD) are characterized by shorter LTL than controls [20,366,367]. Besides LTL, short telomeres were also found in atherosclerotic plaques [45] and their length inversely correlated with atherosclerotic grade and age [29]. In addition, it was shown that cells which build up the

artery wall, such as vascular smooth muscle cells and endothelial cells, undergo senescence induced by short telomeres in atherosclerotic lesions [371,372]. Currently, a debate is ongoing about which of the two events, short telomeres or formation of plaques, is the primary cause. A recent review proposed four hypotheses that could explain the observed short telomeres in atherosclerotic lesions i) short telomeres are inherited; ii) short telomeres are caused by higher attrition rates before ASCVD iii) short telomeres are caused by higher attrition rates during ASCVD; iv) short telomeres observed in blood are an aftereffect of poor telomere maintenance capacity that may also result in local telomere attrition during disease [31].

A growing number of studies are pointing in the direction of short telomeres as determinant of ASCVD. A 9.5-year longitudinal study revealed that shorter LTL at baseline was associated with a higher number of atherosclerotic plaques at follow-up in patients, whereas leukocyte telomere attrition rates were equal in subjects with and without atherosclerotic plaques [373]. Moreover, in this study, the association between short LTL and atherosclerosis was stronger in individuals with early onset of the disease [373]. The authors suggested that individuals with short telomeres may be more prone to early onset and progression to more severe stages of the disease [373]. Indeed, it was shown that individuals with shorter TL had a higher prevalence of ASCVD [374]. Likewise, Mendelian randomization studies based on TL-associated SNPs reported that alleles associated with shorter LTL are over represented in individuals with ASCVD [292,354,364], lending further credence to pre-existence of short TL and a potential causal role in onset of the disease. Moreover, recent knowledge on telomere dynamics from longitudinal and cross-sectional studies in healthy individuals suggest that short LTL in adults could be mainly established during the first decade of life [280,282,316]. However, none of these studies provide mechanistic insights on the role of short telomeres in cardiovascular disease.

## 2.11 Methods for TL measurements

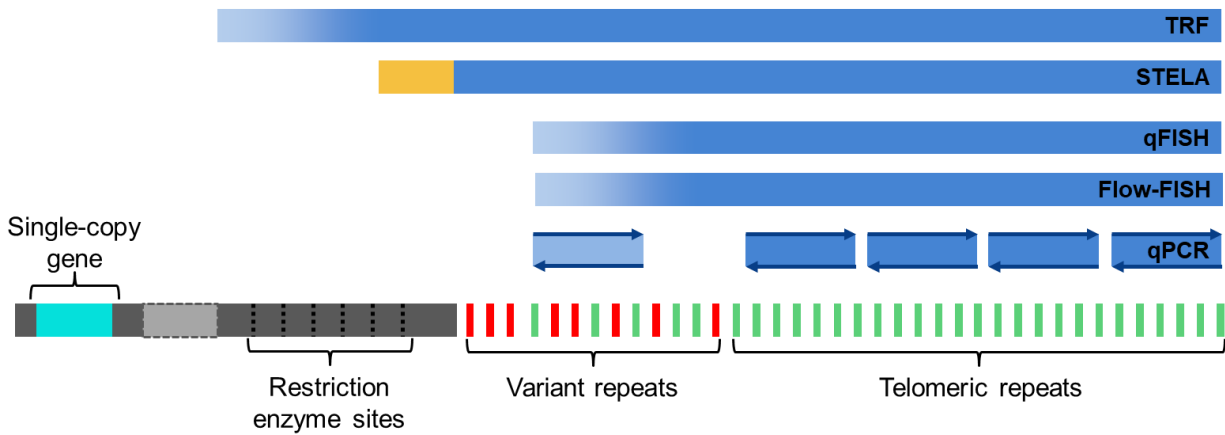
There are several different methods for measuring TL (Figure 12); each one having advantages and caveats. In epidemiological studies with large sample sizes, the two most commonly used methods are quantitative polymerase chain reaction (qPCR) [375] and terminal restriction fragment analysis (TRF) [225,376].

qPCR allows the determination of relative TL by measuring the signal from amplified telomeric repeats normalized to a reference single-copy gene signal [375]. This technique is very fast, has a low cost and requires a low amount of DNA, so it is often applied in high-throughput TL quantifications. However, the measurement error of qPCR can reach up to 28% [377]. Coupled with different reference single-copy genes used in independent laboratories, the results across different studies and laboratories are hardly comparable [378]. In general, a variation in the qPCR results may affect the accuracy of the determination of the difference between TL measured in different subjects, tissues and samples collected with time intervals for longitudinal studies.

The TRF method measures the mean TL in base pairs by Southern blot of the undigested telomeres generated during treatment of genomic DNA with restriction enzymes [225,376]. Depending on the selected cocktail of restriction enzymes, the length of the undigested subtelomere region may vary and affect the mean TL [376]. When the technique is standardized by the restriction enzymes used and the Southern blot protocol, mean TL values can be compared between laboratories. The drawbacks of this technique are that it is expensive, time-consuming, requires a large amount of DNA ( $\sim 3\mu\text{g}$ ) and doesn't detect the shortest telomeres [378].

Regardless, it is considered to be the “gold standard” for TL assessment [378]. In support of this, an impartial comparison of human LTL measurements with the two most commonly used methods, TRF and q-PCR, showed that the TRF method is more reproducible than q-PCR [379]. In addition, it has recently been suggested that the TRF method is suitable not

only for evaluation of mean TL, but also for assessment of the differences between TL distribution using a special computational approach [71].



**Figure 12. Detection of telomere length with different methods (modified from reference [380])**

Blue bars represent DNA regions measured by different methods, while gradient shows regions that have variable detection. The orange box illustrates primer for STELA with known length, which can be subtracted from the calculation of telomere length. Green bars show telomeric repeats and mix of green and red bars shows telomere variant repeats. Grey bar with dash lines illustrate genomic DNA with restriction enzyme sites and light blue is for single-copy gene that is used as a reference for qPCR.

TRF = telomere restriction fragment analysis, STELA = single telomere length analysis, qFISH = quantitative-fluorescence in situ hybridization, Flow-FISH = flow fluorescence in situ hybridization, qPCR = quantitative polymerase chain reaction.

Currently, there are several modifications of the TRF method, such as Single TL Analysis (STELA), Universal Single TL Analysis (U-STELA) and Telomere Shortest Length Assay (TeSLA), which are suitable for the evaluation of ultrashort telomeres [381–383]. Among other techniques, quantitative-fluorescence in situ hybridization (qFISH) and its modification with flow cytometry, Flow-FISH, should be mentioned. qFISH is used to estimate TL in cells or/and in individual metaphase chromosomes [384]. The combination of qFISH with flow cytometry gives the Flow-FISH method [385], which provides a possibility to analyze TL in single cells of the entire population in a short period of time. However, it has some

limitations, such as strong variation of autofluorescence at different levels of population doubling, which require additional steps to improve results [386].

Overall, TL measurement should be carried out using the most appropriate method for each type of experiment, depending on the final purpose and considering all the advantages and disadvantages of the chosen approach. Table 3 provides a comparison of two most used methods in epidemiological studies, TRF and qPCR.

**Table 3. Comparison of TRF and qPCR methods used to measure telomeres in epidemiological studies (modified from reference [376]).**

Method	TRF	qPCR
<b>Materials</b>	1 to 3 µg of DNA per sample	50 ng of DNA per sample
<b>Throughput</b>	Low	High
<b>Advantages</b>	<ul style="list-style-type: none"> <li>• Can measure telomere length distribution</li> <li>• Coefficient of variation &lt; 2%</li> <li>• Measurements expressed in absolute values (kb)</li> </ul>	<ul style="list-style-type: none"> <li>• Low cost</li> <li>• High throughput</li> </ul>
<b>Disadvantages</b>	<ul style="list-style-type: none"> <li>• Labor intensive</li> <li>• Costly</li> <li>• Presence of subtelomeric DNA in TRFs confounds absolute telomeric length estimate</li> </ul>	<ul style="list-style-type: none"> <li>• Only average telomere length is measured</li> <li>• Coefficient of variation &gt; 2%</li> <li>• Lack of good reference standards makes absolute telomere length measurement difficult</li> </ul>



# Hypothesis and objectives

Experimental evidence and parallels between ASCVD and CAVD pathophysiology suggest that mechanisms of valvular calcification may involve pathways that might be activated by short telomeres. Moreover, patients with AS display shorter telomeres in white blood cells in comparison to healthy matched controls. However, the implication of telomere dynamics in aortic valve tissue during AS remains unknown.

We **hypothesized** that telomere length in valves may play a role in the onset and progression of AS through CAVD. Individuals with shorter telomeres may present a higher risk of developing aortic valve stenosis.

The **objective** of this work was to determine the relation between telomere length dynamics in valves and the aortic valve calcification process.

More specifically, the **aims** of the study were as follows:

- I. To explore general characteristics of telomere length dynamics in valves tissue with cross-sectional analysis.
- II. To investigate telomere length dynamics in human aortic valves in individuals with and without AS.
- III. To explore locally telomere dynamics in different areas of valve in valves with macroscopic calcification in both subjects with or without AS.

Telomere dynamics in valves was assessed by mean telomere length, shortest telomere length, telomere length distribution shape and telomerase activity.

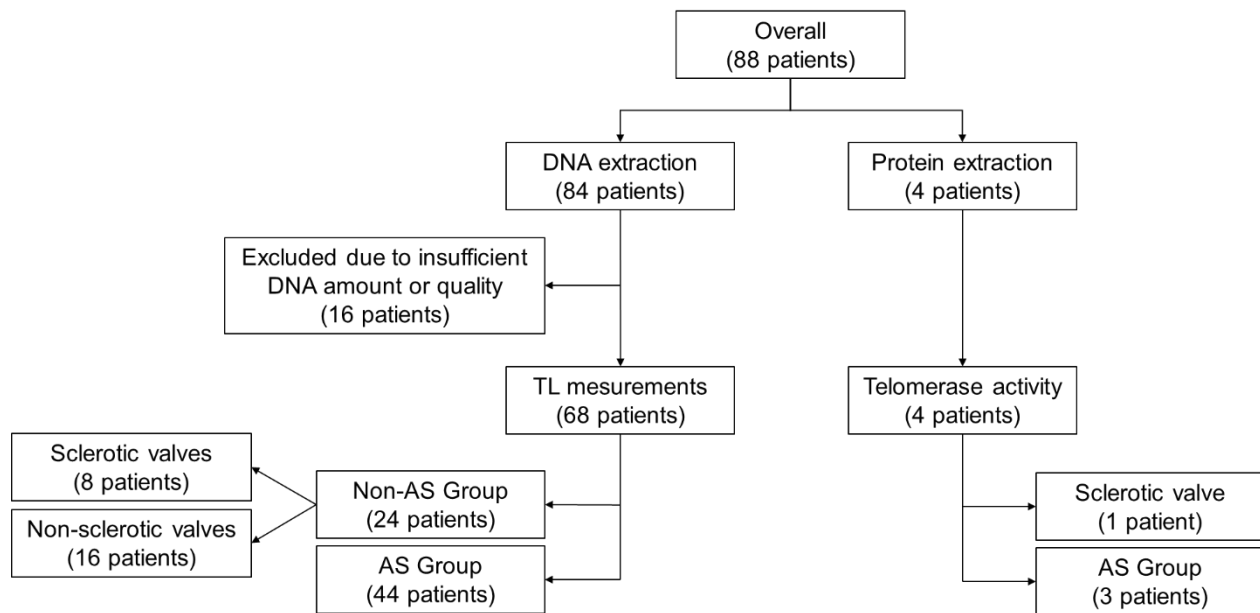
# Methods

## 1 Sample collection

Aortic valves were harvested from patients who had undergone aortic valve replacement surgery for AS, aortic valve regurgitation and aortic dilation at the Karolinska University Hospital in Stockholm, Sweden. All procedures were approved by the local ethics committee (2012/1633). Written informed consent was obtained from all patients. Overall, valves from 88 patients were collected. Both, tricuspid and bicuspid valves were included in the study.

After harvesting, aortic valve leaflets were kept in preservation liquid at 4°C. For TL measurements and transcriptomic analyses, leaflets were stored in RNA Later solution (Qiagen, Venlo, The Netherlands) and for telomerase activity assay, leaflets were handled in DMEM culture media supplemented with 10% fetal bovine serum. At arrival in the lab samples were photographed, dissected, and stored at -80°C for further manipulations.

Among the 88 valves, 84 were collected for TL measurements and 4 for a telomerase activity assay. Sixteen of the patients were excluded from the study due to insufficient DNA amount and/or quality for TL measurements. Thus, only valves from 72 patients were used. For subsequent analysis, individuals were divided into 2 groups according to their diagnosis: patients with AS were included in the AS group, while patients diagnosed with aortic valve regurgitation, and aortic dilation formed the Non-AS group. The Non-AS group was further subdivided into sclerotic and non-sclerotic subgroups depending on the presence of macroscopic signs of calcification in the valves (Figure 13).



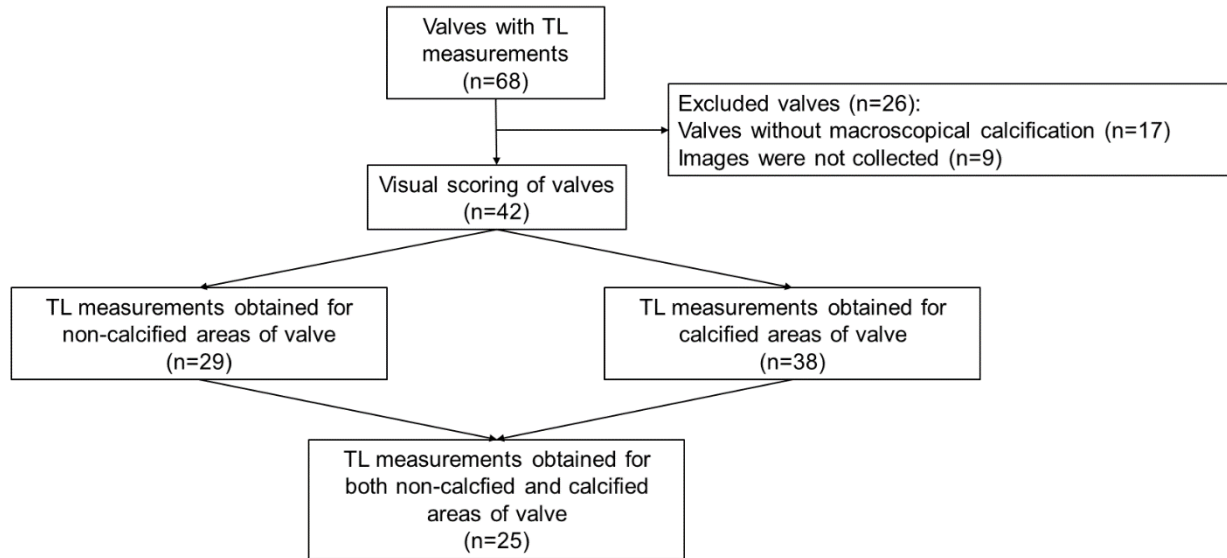
**Figure 13. Flow chart of the study design**  
TL = telomere length and AS = aortic stenosis.

## 2 Assessment of calcification severity by visual scoring of valves

At arrival in the lab, photographs of the valves were taken on a white background. These images were used to assess calcification severity by visual scoring. Figure 14 describes the image selection for the scoring. Macroscopic calcification was distinguished by the presence of visually detectable calcified nodules. For each valve severity of calcification was scored as:

- mild calcification: existence of small areas with calcification, which did not exceed one-third of the valve area;
- moderate calcification: presence of multiple large calcified areas without massive calcification (massive calcification is defined as nodules with big volumes);
- severe calcification: massive calcification of at least one leaflet.

Images were evaluated independently by two observers (observer 1 (Ilona Saraieva) and observer 2 (Oscar Plunde)).



**Figure 14. Flow chart of the visual scoring of valves**  
TL = telomere length.

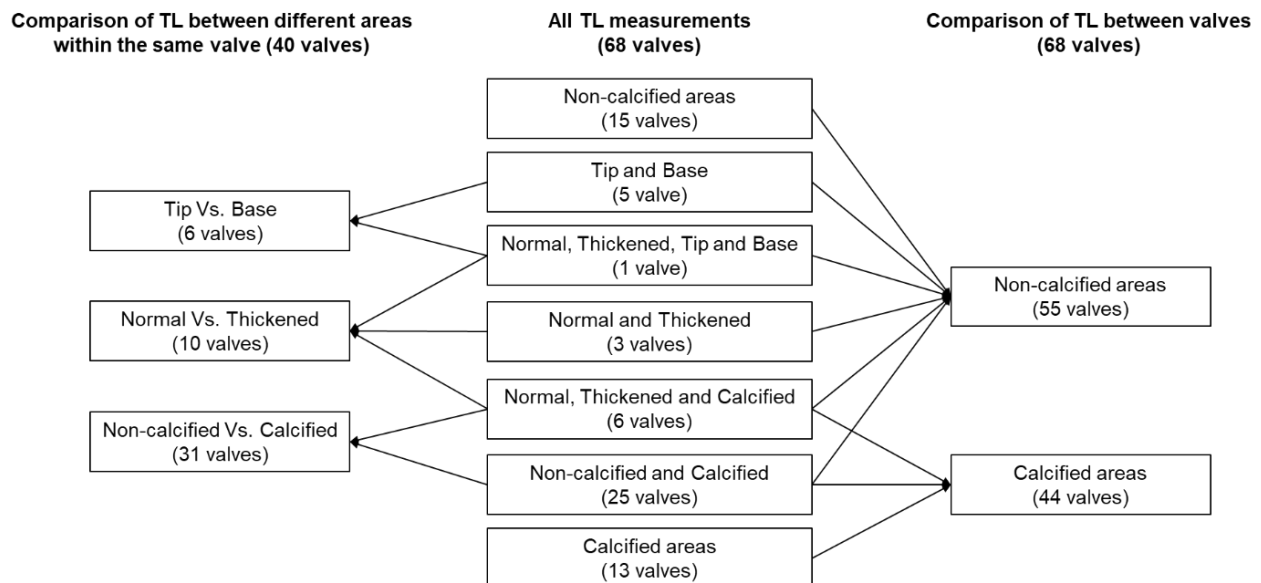
### 3 Macroscopic dissection of aortic valves

Based on the macroscopic evaluation, if possible, aortic valves were dissected into : i) normal (smooth areas without calcifications); ii) thickened (fibrotic areas without clear presence of calcifications) and iii) calcified areas (areas with macroscopic signs of calcifications) as described previously [19]. Note that after we did not find a difference between TL in normal and thickened areas (see results, Figure 29), we combined these two categories of tissues together and called it non-calcified in all subsequent analyses. Additionally, a subset of tricuspid valves without macroscopic calcification was dissected into anatomical areas, namely tip and base. After microscopic dissection, on average, of 1-3 specimens of different tissue categories (normal, thickened, non-calcified, calcified, tip and base areas) were obtained from each valve.

To assess telomere dynamics in aortic valve tissues, we compared TL measurements (both mean TL and mean TL value of 10% shortest telomeres) in different situations (Figure 15). For intra-individual comparison, we used valves with available TL measurements for at least

two tissue areas. We compared TL in 3 situations: normal vs thickened (n=10), non-calcified vs calcified (n=31), and tip vs base (n=6). Valves, which met the criteria for two types of comparison, were included in both. In cases where several samples of one tissue area (normal, thickened, non-calcified, calcified, tip and base) belonged to the same patient, a mean value was calculated.

Valves for which TL measurements were obtained for only one area were not included in the comparisons but used in inter-individual analysis. To assess TL independently of the presence of local calcifications, we used non-calcified areas from valves (n=55) to compare between AS and Non-AS groups.



**Figure 15. Assessment of telomere dynamics in aortic valve tissue according to the samples available**

TL = telomere length

#### 4 DNA extraction protocol

Total genomic DNA was extracted with the phenol/chloroform/isoamyl alcohol method. Each tissue specimen was ground up using a liquid nitrogen precooled BioPulverizer (BioSpec, Bartlesville, OK, USA). Tissue powder was transferred to 5ml tubes. Samples were mixed

with lysis buffer B (75 mM NaCl, 24 mM EDTA (pH 8.0), 0.5% sodium dodecyl sulfate (SDS), RNase A (10 mg/ml) (Thermo Fisher Scientific, Waltham, MA, USA)) keeping a ratio of 1ml of buffer per 200mg of tissue followed by incubation at 37°C for 2 hours. Subsequently, proteinase K (20 mg/ml) (Thermo Fisher Scientific) was added, and the mixture was held at 55°C for 16 hours. The lysate was cooled on ice and then vigorously mixed with equal volumes of phenol/chloroform/isoamyl alcohol (25:24:1) to form an emulsion. The phases were separated by centrifugation at 3000 rpm for 10 minutes at 4°C. The upper, aqueous phase was gently removed and the step with addition of phenol/chloroform/isoamyl alcohol was repeated. The aqueous phase was collected in new tubes containing 1µl of glycogen (5mg/ml) and 1/100<sup>th</sup> volume of 5 M NaCl. Pure precooled ethanol was added to the aqueous phase and gently inverted to precipitate the DNA. Following this step and the next two steps samples were kept on ice. To recover the precipitated DNA, samples were centrifuged, the supernatant discarded, and the DNA pellet was rinsed twice with 70% ethanol. The DNA pellet was dried at room temperature and then dissolved in 50 µl 1xTE buffer (Tris-HCl 10mM pH 7.6 / EDTA 1mM) at 37°C for 2 hours.

Total DNA concentration was calculated from 260 nm absorbance using a Nanodrop spectrophotometer (Thermo Scientific), and purity was assessed by the 260 nm/280 nm optical density ratio. Integrity of DNA was assessed by visualization on 1% (wt/vol) agarose gel using ethidium bromide staining. Agarose gel electrophoresis was carried out by loading 10 µl of DNA (20ng/ µl) mixed with 2.5 µL of 10x loading dye (Thermo Fisher Scientific) onto an agarose gel. Samples were run for approximately 45 min at 100 V. UV visualization of DNA bands on the gel was performed using the GelDoc imager (Biorad, Hercules, CA, USA). DNA with signs of degradation (shift downward of the DNA “crown” or smear under the crown) was considered unfit for analyses. All samples were stored at -80° C until further use.

Extraction of DNA according to the following protocol resulted in highly contaminated DNA obtained from valve samples, thus several modifications were applied to get rid of impurities.

#### **4.1 Modification 1 of DNA extraction protocol**

Cold temperature during extraction facilitates precipitation of salts together with DNA. After addition of pure precooled ethanol, samples were gently mixed till the concentrated DNA was observed visually. To avoid further co-precipitation of DNA with impurities it was captured with a pipet tip and transferred into a new tube containing 70% ethanol as fast as possible.

This modification did not lead to complete purification of the samples from impurities. Thus, one more step was applied.

#### **4.2 Modification 2 of DNA extraction protocol**

To purify DNA from contamination, each DNA sample was reprecipitated at least two times. The DNA sample was mixed with 500  $\mu$ L of lysis buffer B and 20  $\mu$ l of 5M NaCl and then precipitated with 1.25 mL of pure precooled ethanol. The DNA pellet was recovered as described above. The second reprecipitation step was similar to the first, except for the use of 1xTE buffer instead of the lysis buffer B.

This allowed us to obtain non-contaminated DNA.

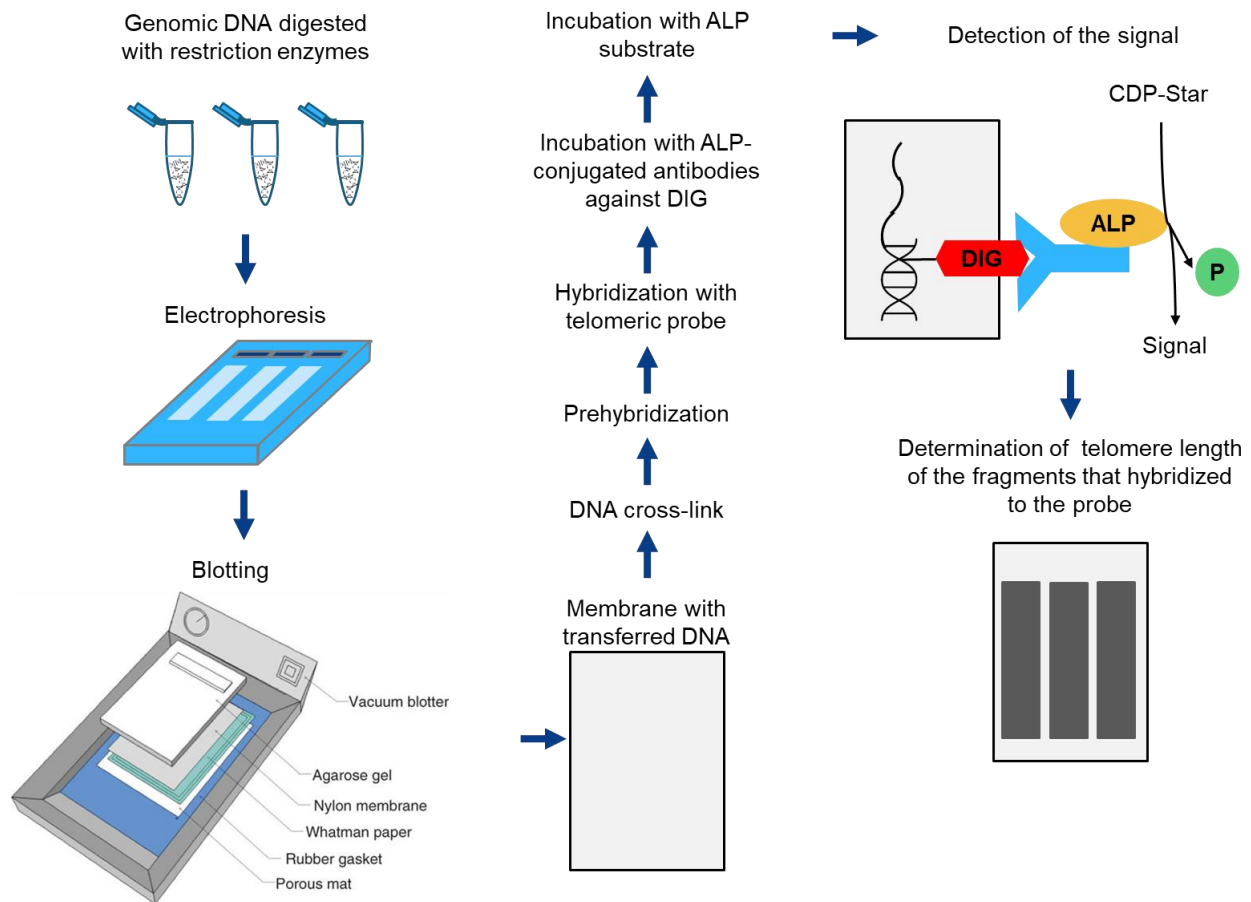
### **5 Telomere length measurements: TRF method**

TL measurements were performed by Southern blot analysis of the terminal restriction fragments (TRF) (Figure 16) using products from the TeloTAGGG™ Telomere Length Assay kit (Roche Diagnostics GmbH, Mannheim, Germany) with a modified protocol (adapted from Kimura et al [376]). Briefly, 3 $\mu$ g of DNA samples were digested with restriction enzymes HinfI (10U) and RsaI (10U) for 16 hours at 37°C. Digested DNA samples and DNA molecular weight ladders were separated in 0.5% (wt/vol) agarose gels for 24h at 40V in 0.5x TBE buffer. After electrophoresis, DNA was depurinated on the gel by

incubation with 0.25 M HCl, denatured with a solution of NaOH and NaCl (0.5 M and 1.5 M, respectively) and neutralized with a solution of NaCl and Tris (1.5 M and 0.5 M respectively). Subsequently, DNA was transferred by vacuum blotting onto positively charged nylon membranes and cross-linked by UV light. After one hour of prehybridization, the membrane was hybridized with a digoxigenin-labeled telomeric probe at 42°C overnight. The membrane was then washed twice with 2x saline sodium citrate (SSC)/0.1% SDS at room temperature, followed by two wash steps with 0.2 x SSC/0.1% SDS at 50°C to eliminate non-specific probe binding, blocked for proteins and incubated with a digoxigenin-specific antibody bound to ALP. Probe-target hybrids were detected using ALP conjugate hydrolysis of a chemiluminescent CDP-Star substrate solution (Roche Diagnostics GmbH). Chemiluminescence of metabolized CDP-Star was visualized by CCD camera (Las 4000, Fujifilm Life Sciences, Cambridge, MA, USA). Average TL was calculated based on signal intensity of the distributed fragments and their sizes that was estimated using a molecular weight ladder [376].

Different samples from the same individual were run in adjacent lanes on the same membrane. Duplicate measurements on separate membranes were run for samples with sufficient amounts of DNA. Overall inter-assay coefficient of variation for the duplicate measurements was 2.07%.





**Figure 16. Schematic flow-chart of telomere restriction fragment analysis**

DIG = digoxigenin, ALP = alkaline phosphatase, P = phosphorus. Image of vacuum blotter is taken from reference [376].

## 6 TL distribution computation

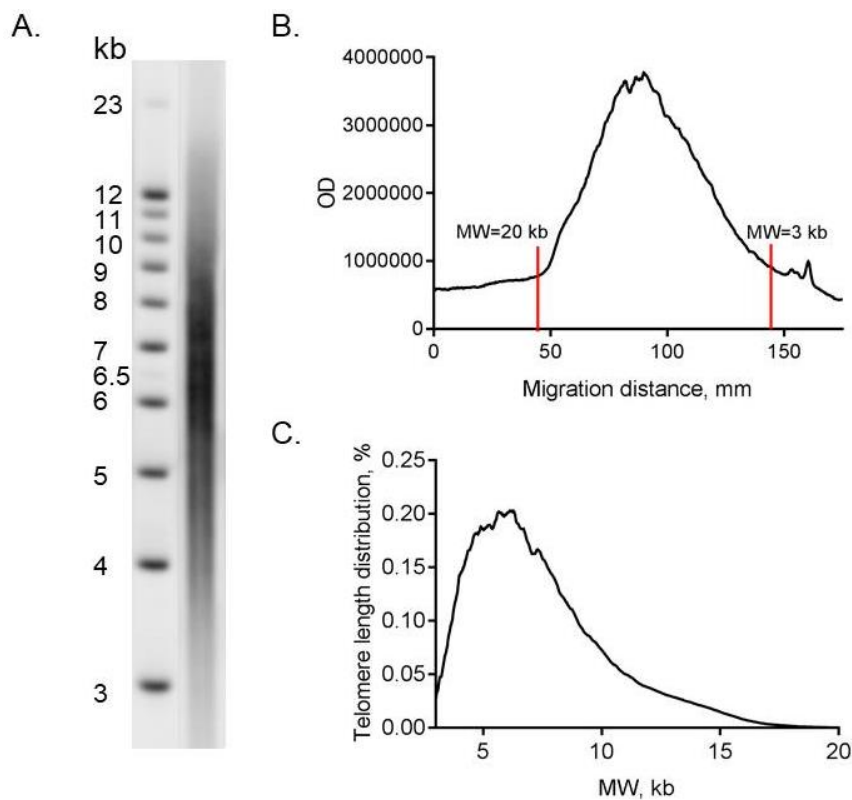
The TRF measurement method gives access to the whole distribution of TL in one sample (Figure 17A and B) but usually only mean TL calculations were generated from raw data and used in the analyses. In collaboration with Dr. Emma Horton, Pr Anne Gegout-Petit and Dr Denis Villemonais (Université de Lorraine, CNRS, Inria, IECL, F-54000, Nancy, France) we studied TL distribution (TLD) characteristics. Mathematical computations allowed us to obtain whole distribution of TL in one sample from the raw data generated by the TRF method measurement. The calculations were performed as previously described [71]. In

brief, for each sample TLD was obtained in the range from 3 kb to 20 kb using the following formula:

$$\text{TLD} = \frac{\text{OD}/\text{MW}}{\sum \text{OD}/\text{MW}}, \text{ where OD is optical density and MW is molecular weight}$$

The distribution represents the proportion of each telomere of length MW(x) in the 3-20 kb window (Figure 17C).

Obtaining TLD for each sample allowed new type of comparison and analyses.



**Figure 17. Representative example of telomere length distribution in valve sample**

A, Telomere length distribution detected by Southern blot. B, Distribution of the optical density along the membrane derived from A. C. Proportion of each telomere of length MW(x) in the 3-20 kb window.

OD = optical density and MW = molecular weight.

## 6.1 Comparison of TLD shape using Kolmogorov distances

First, it was possible to compare TLD shape (that is representative of the distribution of length observed in the sample). To this end, other mathematical steps were required. Following TLD obtention, TLD were all translated to examine the difference in shape without the influence of the mean TL. The cumulative distribution function (CDF) was calculated on translated TLD. To this end, the translated TLD function was partitioned into 1000 equally sized segments and for each MW value in the partition, the proportion of values that were less than MW in the DNA sample was obtained:

$$CDF = \sum_{MW' \leq MW} TLD(MW')$$

Next, the Kolmogorov distance (KD) was used to assess the maximum difference between the CDF of two distributions. This allowed us to compare the CDF between and within patients. To simplify, the more difference in shape existing between 2 TLD, the higher is the calculated KD.

## 6.2 Analysis and comparison of shortest telomeres of the TLD

In addition, obtention of TLD allowed us to examine the shortest telomeres of the TLD and their possible implications in disease. We focused on 10% of the shortest telomeres of the distribution and calculated the mean length of these telomeres. For this, the TLD function was partitioned into 10 equally sized segments (equal proportion of telomeres in each segment) and the 1<sup>st</sup>-quantile was used to calculate the mean of this TLD segment, which corresponded to the mean TL value of the shortest 10% of the whole TL distribution [71].

## 7 Telomerase activity

Telomerase activity was determined using the telomere repeat amplification protocol (TRAP) assay as described previously [387]. Briefly, the TRAP assay consists of three main steps:

telomerase substrate (TS) primer extension by telomerase, amplification and detection of telomerase products (Figure 18). In the extension step, telomerase, which is present in cell extracts, adds telomeric repeats to TS, an artificial non-telomeric oligonucleotide. The telomerase products are then amplified by a subsequent PCR reaction using TS oligonucleotide as the forward primer and an oligonucleotide that is complementary to telomeric repeats as the reverse primer (CXext) [388]. PCR products are detected by polyacrylamide gel electrophoresis followed by SYBR Green I staining and UV-exposure.

## **7.1 Sample preparation**

Proteins were extracted from non-calcified and calcified areas of valves using 1xCHAPS buffer (10mM Tris (pH 7.5), 1mM MgCl<sub>2</sub> 1mM EGTA, 0.5% CHAPS, 10% glycerol) after grinding the tissues in liquid nitrogen. Lysates were kept for 30 min on ice. After the incubation, lysates were centrifuged at 12 000 rpm for 20 min at 4°C. The supernatant was recovered and protein concentrations were measured using the Bradford assay [389]. Briefly, 2 µl of protein extract were diluted in 498 µl of pure water. The diluted sample was mixed with an equal volume of Bradford reagent (Quick Start™ Bradford Protein Assay, Bio-Rad laboratories, USA) and well mixed. After 5 min of incubation, 300 µl of the mix was added into a 96-well plate (Greiner Bio-One, Germany). Optical density was measured with a spectrophotometer (FLUOstar optima, BMG Labtech, Ortenberg, Germany) at 595 nm. The concentration of the samples was determined using a standard curve of 6 points with known concentrations measured concurrently. Each sample was performed in triplicate. For further usage, samples were diluted in 1xCHAPS buffer to a concentration 0.5 µg/µl.

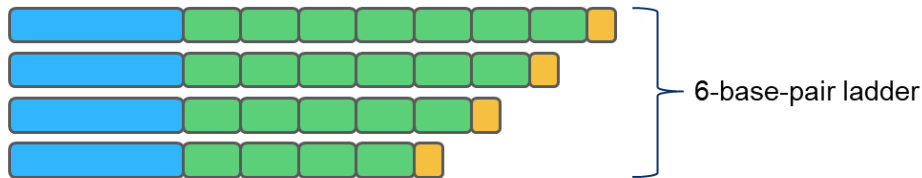
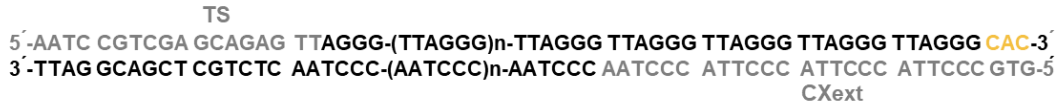
## Extension step



Telomerase

## Amplification step

Hybridization of CXext primer to telomerase product and ladder generation



Formation of primer-dimer (TS-CXext)



## Detection step

Separation of the products by electrophoresis in polyacrylamide gel and detection

### Figure 18. Telomerase activity assay (adapted from reference [388])

Telomerase adds TTAGGG repeats to TS primer. Telomerase products are amplified during PCR reaction using CXext primer as a reverse. Special design of CXext primer prevents elongation of the repeats by capping them with non-telomeric CAC sequence. The PCR leads to the formation of a 6-base-pair ladder that reflects processivity of telomerase and also the minimal TS-CXext primer-dimer product. Subsequent detection step allows visualizing these products. TS primer is shown in green, CXext primer is shown in blue, telomeric repeats are shown in black and CAC sequence is shown in orange colours.

## 7.2 TRAP with ITAS

Prior to starting the TRAP reaction, the master mix was prepared. For each sample 50 µl reaction mix was made by mixing 48 µl of master mix with 2 µl of the protein extract. Final composition of components were as follows: 1xTRAP buffer (20 mM Tris-HCl (pH 8.3), 1.5

mM MgCl<sub>2</sub>, 63 mM KCl, 1 mM EGTA, 0.005% Tween 20), 22.5 pmol primer TS, 22.5 pmol primer CXext, 1 pmol primer NT, 0.01amol primer TSNT, 50 μM dNTPs, 2 units of Taq-polymerase and 1μg of proteins. Of note, primer TSNT was used as internal telomerase assay standard (ITAS) and amplified in PCR by TS (forward) and NT (reverse) primers [390]. Sequences of primers used in the TRAP reaction are presented in Table 4. Samples were placed at 37 °C for 30 min for telomerase mediated extension of TS primer followed by the PCR reaction. The initial step of PCR included Taq-polymerase activation and telomerase deactivation for 2 min at 94°C and then the PCR was cycled 33 times at 94 °C for 30 s, 52 °C for 30 s and 72 °C for 20 s with a final extension step of 10 min at 72°C. Next, 2 μL of 10x BlueJuice gel loading buffer (Invitrogen, Eugene, Oregon, USA) was added to 18 μl of PCR products and loaded on a 12% non-denaturing polyacrylamide gel (19:1). Electrophoresis was performed at 80 V for 30 min and then followed by 180V for around 45 min until bromophenol blue ran out of the gel. Gels were then stained with 1xSYBR Green I (Invitrogen) for 10 min. DNA bands were visualized with UV on a GelDoc imager (Biorad).

**Table 4. Primers used in TRAP assay**

<b>Primer</b>	<b>Sequence (5' to 3')</b>
<b>TS</b> (forward for telomerase product and for TSNT)	AATCCGTCGAGCAGAGTT
<b>CXext</b> (reverse for telomerase product)	GTGCCCTTACCCTTACCCTTACCCTAA
<b>TSNT</b> (internal control)	ATTCCGTCGAGCAGAGTTAAAAGGCCGAGAAGCGAT
<b>NT</b> (reverse of TSNT)	ATCGCTTCTCGGCCTTTT

### **7.3 Modification of the TRAP**

Considering that in some cases TSNT primer can be amplified in presence of PCR inhibitors, which results in false positive results [391], we also performed modified version of TRAP reaction without ITAS. Basically, reaction was carried out in the same conditions as TRAP with ITAS, except primers TSNT and NT were not added into the PCR mix.

### **7.4 Selection of Taq-polymerase**

Two different types of Taq-polymerase were used in the TRAP assay: Taq-polymerase from *Thermus aquaticus* (Roche, cat. number 11 146 173 001) and FastStart Taq-polymerase (Roche, cat. number 12 032 929 001).

### **7.5 Assessment of the annealing temperature**

The effect of annealing temperature on Taq-polymerase processivity and primer-dimer formation was estimated in a temperature gradient from 52°C to 60°C.

### **7.6 Control reactions**

To avoid false-positive and false-negative results, control reactions were assayed for each sample. To ensure that the observed amplified products of PCR were dependent on telomerase activity in the first step, reactions with heat-inactivated telomerase at 95°C for 10 min were performed. To rule out false-negative results, a second control reaction was performed, containing 60 ng of protein extract from HeLa cells mixed with each sample of interest before subjecting it to the TRAP reaction. We considered that sensitivity of the method was at least equal to 60 ng of total protein extract from HeLa cells, if the signal that corresponded to telomerase activity appeared in these false-negatives controls.

## **8 RNA Extraction and Transcriptomic Analysis**

A transcriptomic analysis was performed by Oscar Plunde in the laboratory of Prof. Magnus Back in Stockholm. Briefly, total RNA from 11 pieces of aortic valve tissue from 8 patients was extracted using the TissueLyzer and RNeasy Tissue Mini Kit (Qiagen). A NanoDrop (Thermo Scientific) was used to quantify RNA concentration and quality was assessed using 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA). Total RNA levels were measured with an Affymetrix Human Transcriptome 2.0 (HTA 2.0 arrays, Santa Clara, CA, USA) via the core facility for Bioinformatics and Expression Analysis at Karolinska Institute. Normalization of the data was performed with Signal Space Transformation-Robust Multi-Chip Analysis and standard quality control was carried out using Transcriptome Analysis Console Software (ThermoFisher Scientific). The expression data was log<sub>2</sub> transformed. In a following step, batch and tissue type adjusted data were visualized with principal components in Qlucore Omics Explorer 3.4 bioinformatics software (<http://www.glucore.com>). Principal component analysis (PCA) was performed using covariance matrix on the raw data set without any filtering or statistical test applied.

For data evaluation, we divided samples into two groups according to their TL. The division was performed across the median TL of 11 samples, 5 samples with TL below 7.69 kb were considered as those with short telomeres and 6 samples with TL above 7.69 kb were considered as those with long telomeres. Overall, TL in these samples ranged from 7.08 kb to 8.01 kb

## **9 Statistical analysis**

Results are expressed as percentages for discrete variables and mean  $\pm$  standard deviation, or median  $\pm$  interquartile range for continuous variables according to their normality. Comparisons between groups were performed using a two-sample t-test or the Mann-Whitney test as appropriate for continuous variables and  $\chi^2$  tests for discrete variables. We



used paired t-test or the Wilcoxon signed-rank test according to their normality for intra-individual comparisons of different tissue areas. We used Pearson's correlations to test bivariate relations between continuous variables. Adjustments for age and sex were made using a general linear model.

Comparison between calcification severity groups was assessed by one-way ANOVA with trend analysis. The Cohen's kappa coefficient was calculated to measure intra- and inter-observer variability. Inter- and intra-observer agreement was interpreted according to the scale: excellent ( $\geq 0.80$ ), good (0.60-0.79), moderate (0.40-0.59), poor (0.20-0.39) and very poor ( $<0.20$ ) [392].

The Mann-Whitney test and the Kruskal-Wallis test with Dunn's test as a post-hoc analysis were used for mean KD comparison between groups. It should be mentioned that these tests do not take into account that the data are dependent. However, a statistical test suitable to compare two groups with dependent variables, the Mantel test, is usually applied to square matrices and in our study, the number of samples in groups varies and does not fulfill criteria for the Mantel test. Thus, we chose to apply the Mann-Whitney and the Kruskal-Wallis tests. Therefore obtained  $p$  values in these comparisons should be treated with caution.

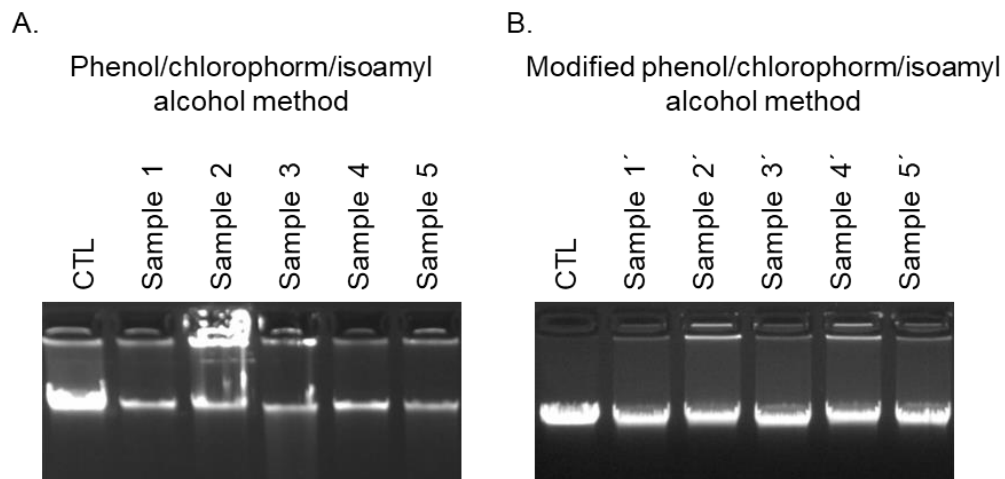
All statistical analyses were performed using NCSS 9 (NCSS, Kaysville, UT), GraphPad Prism 8.4.0 (GraphPad, San Diego, CA, USA) and R Statistical Software (R Foundation for Statistical Computing, Vienna, Austria). Significance level was set at  $p$  value  $<0.05$ .

# Results

## 1 Optimization and modification of the methods

### 1.1 Modification of the DNA extraction method

DNA extraction from valve samples with the standard phenol/chloroform/isoamyl alcohol method resulted in highly contaminated DNA. The 260nm/280nm optical density ratio for extracted DNA ranged from 1.4 to 2.0, which indicates the presence of impurities (protein, phenol, or organic contaminants). DNA contamination contributed to an overestimation of the DNA concentration by spectrophotometry that was detected during DNA electrophoresis for the integrity check (Figure 19). Indeed, DNA amount used for the integrity check should be the same in each lane (based on estimation by spectrophotometry) and differences in amounts were visible. Moreover, these impurities inhibited endonucleases used for DNA digestion during TRF measurements (Figure 20).

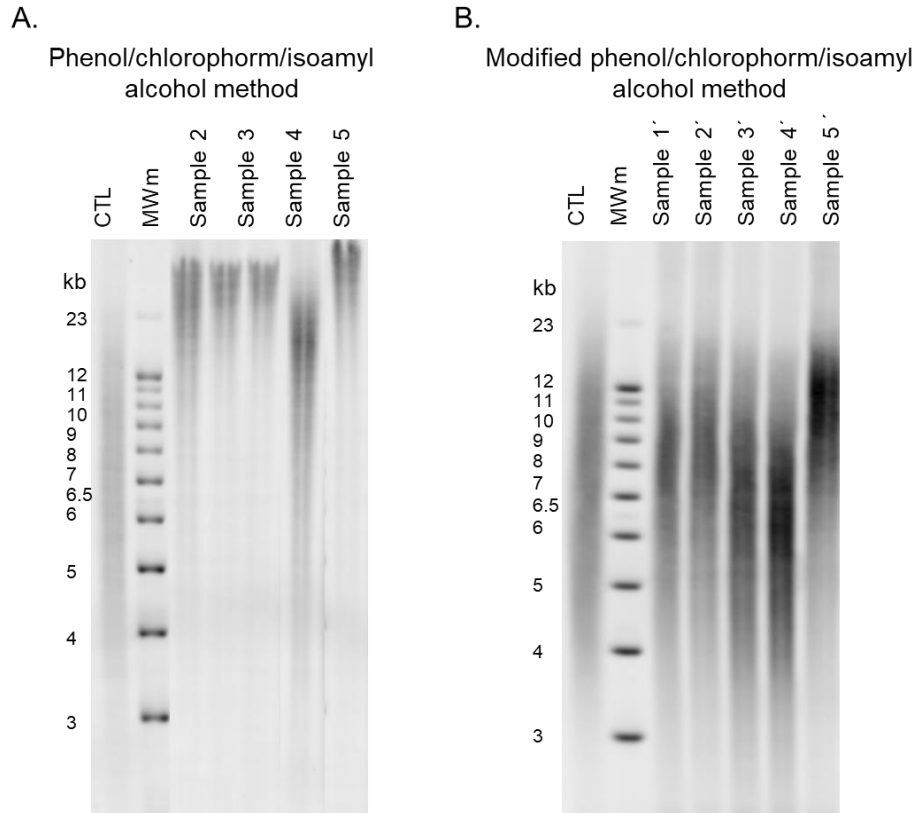


**Figure 19. UV-visualization of DNA samples on agarose gel after electrophoresis**

Differences in DNA amount are visible with classic protocol (A). With modified protocol, all samples display the same intensity indicating the same amount of DNA (B).

CTL = control.

Several changes in the DNA extraction protocol, such as avoiding long time exposure of tissue lysates to cold temperature during mixing of the sample with pure ethanol followed by several rounds of DNA reprecipitation, improved quality of the isolated DNA. Indeed, the 260/280 ratio range became 1.8-1.9 and there was no more interference with DNA concentration estimation (Figure 19) or with endonuclease digestion (Figure 20).



**Figure 20. Representative illustrations of Southern blots showing the TRF of DNA samples extracted with different protocol modifications**

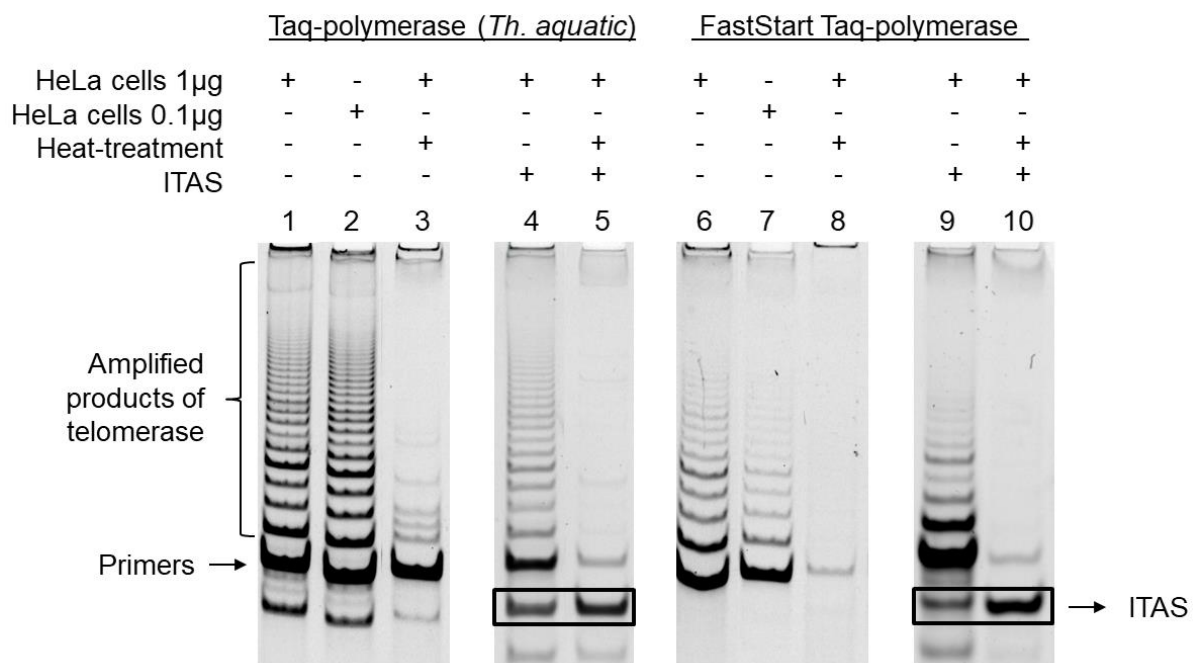
Undigested DNA samples are visible with classic protocol (A) With modified protocol, samples were digested with restriction enzymes (B).

MWm = molecular weight marker; CTL = control; kb = kilobase.

## 1.2 Establishment of TRAP assay

Different conditions for the telomerase activity assay were compared to determine the optimal one in terms of specificity (lower generation of false-positive results) and sensitivity (lower generation of false-negative results).

To enhance specificity, we performed different set-ups of the TRAP assay (with and without ITAS) using serially diluted HeLa cell extracts as positive controls and different qualities of Taq-polymerases. We observed a lower intensity of the signal in reactions with ITAS (Figure 21, line 1 versus line 4 and line 6 versus line 9). Furthermore, we found that using Taq-polymerase from *Th. aquaticus* resulted in generation of primer-dimer artifacts in negative controls (Figure 21, lines 3 and 5). Serial dilution of HeLa cell extract didn't affect telomerase activity in the TRAP assay with this Taq-polymerase (Figure 21, lane 1 and 2). In contrast, a decrease in signal of amplified telomerase products was observed while lower amounts of HeLa cells extract were subjected to the reaction with FastStart Taq-polymerase (Figure 21, lanes 6 and 7). In addition, no primer-dimer artifacts were detected with this Taq-polymerase (Figure 21, lanes 8 and 10).

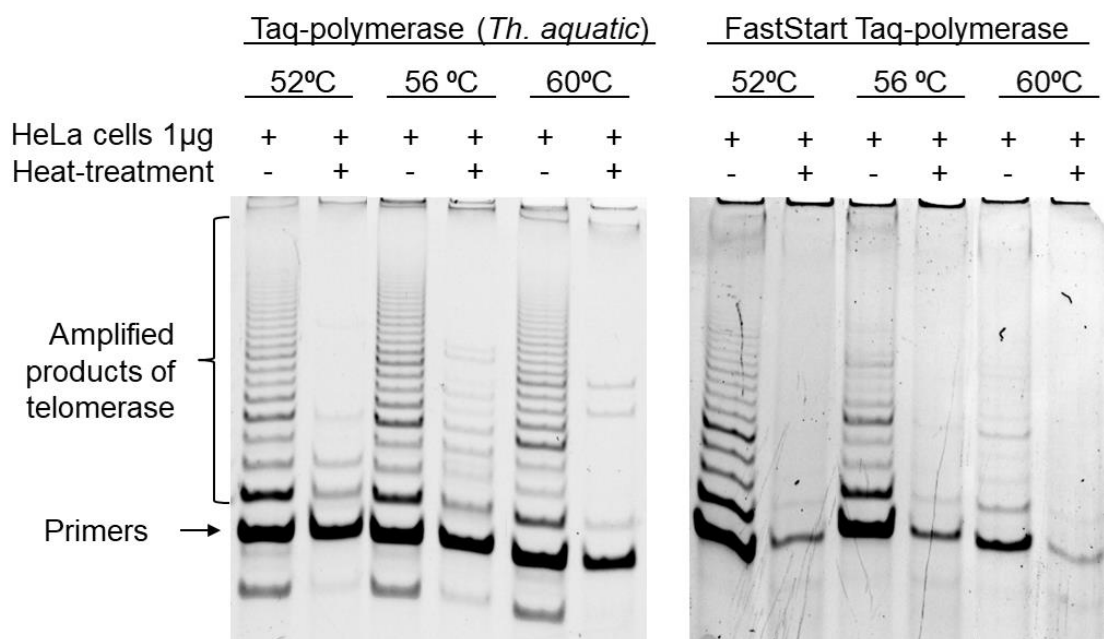


**Figure 21. Optimization of the conditions for telomerase activity assay**

TRAP assay was performed with different Taq-polymerases: Taq-polymerase from *Thermus aquaticus* and with FastStart Taq-polymerase. Two modifications of TRAP assay (with and without internal telomerase assay standard) were analysed. Serial dilutions of HeLa cells extracts were subjected to the TRAP assay. Lines with heat-treated HeLa cells extract represent negative controls. Bands in black boxes correspond to internal telomerase assay standard.

ITAS=internal telomerase assay standard.

Increasing the temperature of the annealing step of the PCR reaction revealed no change in the 6-base-pair ladder signal in TRAP with Taq-polymerase from *Th. aquatic*, whereas signal in reactions performed with FastStart Taq-polymerase negatively correlated with annealing temperature (Figure 22). In addition, abundant generation of primer-dimer artifacts was observed in negative controls of TRAP assay with Taq-polymerase from *Th. aquatic*. Therefore, to obtain the best specificity of the methods we chose the following conditions: i) TRAP assay without ITAS; ii) FastStart Taq-polymerase and iii) 52° C during annealing step of PCR.

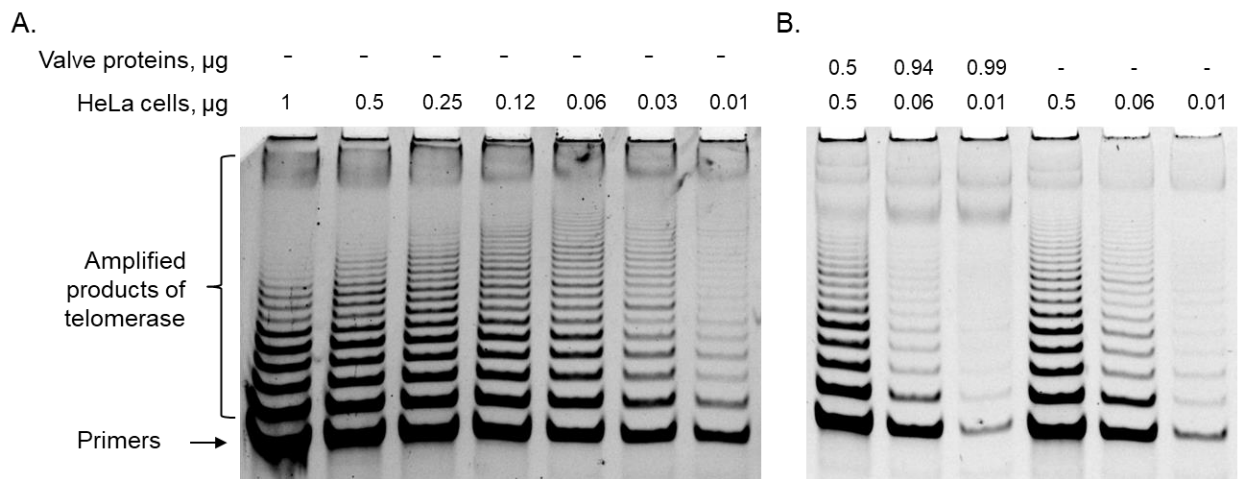


**Figure 22. Gradient PCR during TRAP performance**

TRAP assay was performed with different Taq-polymerases: Taq-polymerase from *Thermus aquatic* and with FastStart Taq-polymerase. Lines with heat-treated HeLa cells extract represent negative controls.

We performed also a modification of the TRAP protocol to define the sensitivity of our test. To achieve this, we performed the TRAP reaction with serial dilution of Hela extracts. In chosen conditions we were able to detect telomerase activity in the range of 1-0.01 µg of HeLa extract (Figure 23A).

Previously, it was shown that tissues may contain inhibitors of the TRAP reaction [393]. Thus, to avoid false-negative results, we established additional control reactions for each tested sample. This reaction included a mix of HeLa and valve protein extracts. Figure 23B shows that no telomerase products were obtained when mixing 10 ng of HeLa extract with 990 ng of valve protein extract, while the reaction with 60 ng of HeLa extract was slightly inhibited but still produced a signal. Following this, we used additional controls for each tested sample with 60 ng of HeLa extract. We considered that sensitivity of the TRAP reaction in valve protein extracts was equal to telomerase activity in 60 ng of total protein extract from HeLa cells.



**Figure 23. Sensitivity of TRAP assay achieved by measuring telomerase activity with different amount of HeLa extract**

TRAP assay was performed with different amount of HeLa extract (A). TRAP assay performed with mixed samples of HeLa extract and valve proteins and with HeLa extract only (B).

## 2 Telomere dynamics in aortic valves

### 2.1 General characteristics of the cohort

Overall, aortic valves from 72 patients were included in this work, of which 68 were collected for TL measurement and 4 for telomerase activity analysis. The subjects were mostly men (77.8%) with a mean age of 64.8 years. Among these 72 patients 43.1 % displayed tricuspid valve, 76.4% had CAVD and 65.3% were diagnosed with AS. TL in non-calcified areas of the valves, measured for 55 patients, was  $8.59\pm 0.68$  kb, while in calcified areas, measured for 44 patients, was  $7.99\pm 0.72$  kb. Characteristics of the patients are presented in the Table 5.

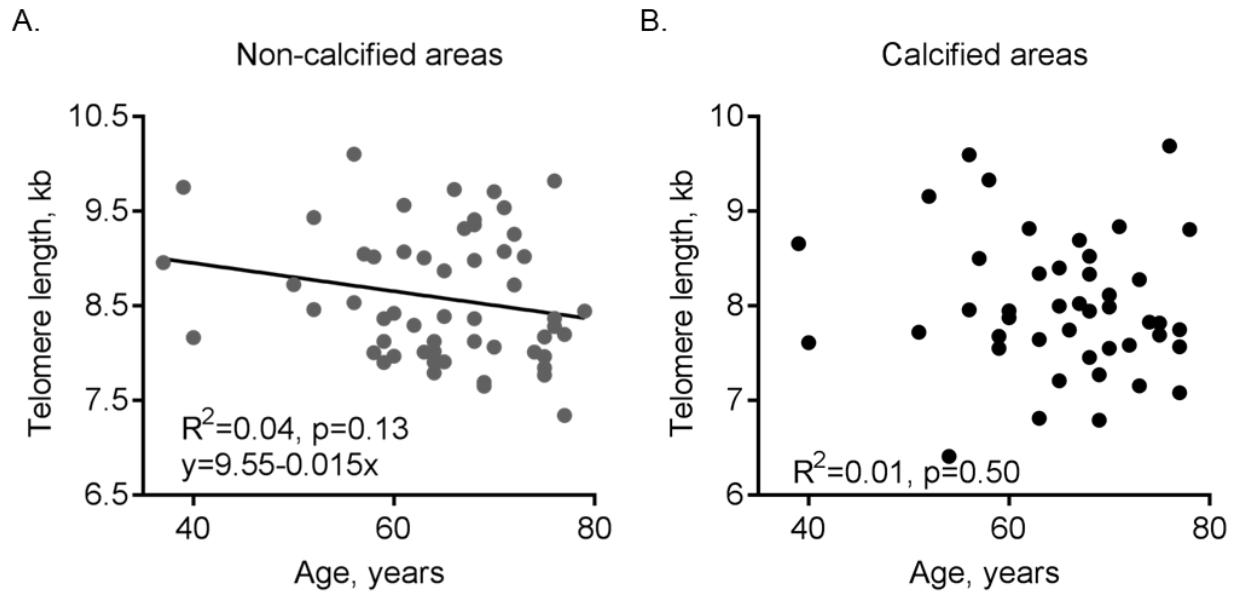
**Table 5. Characteristics of the patients**

	Mean $\pm$ SD or %
Age, (years)	64.8 $\pm$ 10.2
Men (%)	77.8
Tricuspid valve (%)	43.1
CAVD (%)	76.4
AS (%)	65.3

Values are expressed as mean $\pm$ SD or percentage

### 2.2 Age effect on telomere dynamics in aortic valve

TL shortening is associated with aging in different tissues, thus we first investigated possible age effect in our cohort. There was a trend toward shorter telomeres in non-calcified areas of valves with age ( $p=0.13$ ; Figure 24A). The rate of TL shortening was equal to 15 base pairs per year. No association was found between age and TL in calcified valve areas (Figure 24B). Of note, adjustment by sex had no impact on age effect on TL (non-calcified areas:  $p=0.31$ , calcified areas:  $p=0.74$ ).



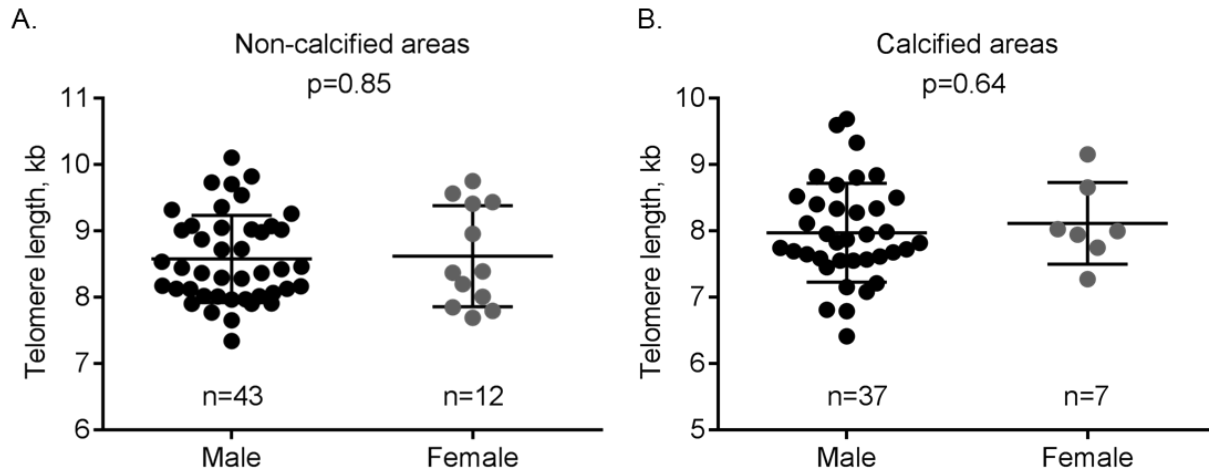
**Figure 24. Association of age with telomere length in non-calcified (A) and calcified areas of valve (B)**

Statistical analysis was performed using Pearson correlation.

### 2.3 Sex effect on valve telomere dynamics in aortic valve

We also investigated whether sex had an effect on valve TL. TL did not differ between males and females in non-calcified valve areas ( $8.58 \pm 0.66$  kb versus  $8.62 \pm 0.76$  kb,  $p = 0.85$ ; Figure 25A). Similar results were found for calcified valve areas ( $7.97 \pm 0.75$  kb versus  $8.11 \pm 0.62$  kb,  $p = 0.64$ ; Figure 25B). Adjustment for age had no impact on these estimates (non-calcified areas:  $p = 0.95$ , calcified areas:  $p = 0.70$ ).



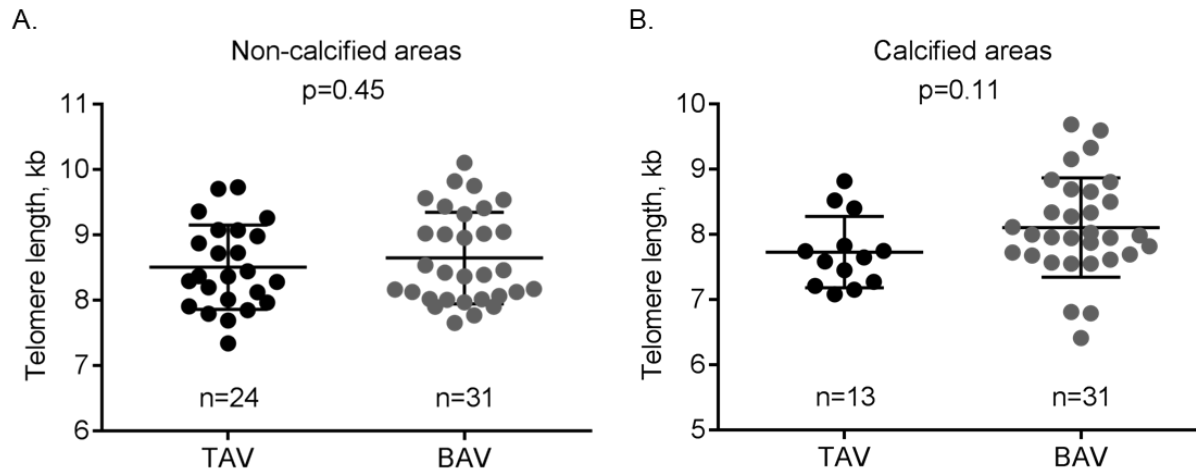


**Figure 25. Association of sex with telomere length in non-calcified (A) and calcified areas of valve (B)**

Data are presented as mean $\pm$ SD. Statistical analysis was performed using two-sample t-test.

#### 2.4 TL in patients with bicuspid and tricuspid valves

It is known that subjects with bicuspid valves develop calcifications one decade earlier than patients with tricuspid valves [11]. Thus, we examined whether presence of congenital abnormalities was associated with shorter TL. In our cohort, patients with bicuspid valves were 7.3 years younger than those with tricuspid valves ( $62.1\pm 10.1$  years and  $69.4\pm 6.3$  years respectively,  $p=0.001$ ). However, no difference was found between TL in non-calcified areas of bicuspid and tricuspid valves ( $8.65\pm 0.70$  kb versus  $8.51\pm 0.65$ ,  $p=0.45$ ; Figure 26A). These results did not change after adjustment for age and sex ( $p=0.91$ ). Similarly, TL in calcified areas did not differ in bicuspid and tricuspid valves ( $8.10\pm 0.76$  kb versus  $7.73\pm 0.55$  kb,  $p=0.11$ ; Figure 26B). Additional adjustment for age and sex had no effect on the results ( $p=0.14$ ).



**Figure 26. Telomere length in non-calcified (A) and calcified areas (B) of tricuspid and bicuspid valves**

Data are presented as mean±SD. Statistical analysis was performed using two-sample t-test.

TAV = tricuspid aortic valve and BAV = bicuspid aortic valve.

## 2.5 Characteristic of patients according to the AS diagnosis

Overall, TL in non-calcified valve areas was measured for 55 patients. These subjects were mostly men (78.2%) with a mean age of 64.7 years. Thirty-two patients, who underwent aortic valve replacement surgery due to AS, were included in AS group. All of them but one had valves with macroscopic calcification. The additional 23 patients, who underwent aortic replacement surgery due to aortic valve regurgitation and aortic dilation, composed the Non-AS group. Seven patients from this group displayed macroscopic calcification in the valves. The 2 groups did not differ in age and sex, while the proportion of tricuspid valves was higher in the Non-AS group. Characteristics of the patients are presented in the Table 6.

**Table 6. Description of the patients according to AS diagnosis**

	<b>AS group</b>	<b>Non-AS group</b>	<b>P</b>
	<b>n=32</b>	<b>n=23</b>	
<b>Age, years</b>	63.3±10.0	66.7±8.6	0.21
<b>Men (%)</b>	75.0	82.6	0.50
<b>Tricuspid valve (%)</b>	28.1	65.2	<0.01
<b>Macroscopical calcification (%)</b>	96.9	30.4	<0.0001

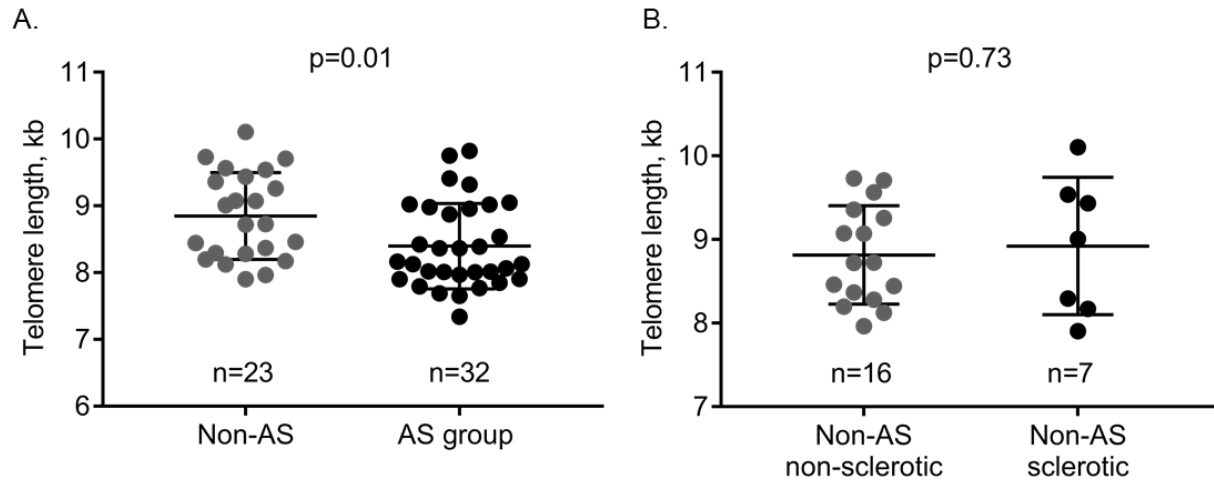
Data are presented as mean±SD or percentage.

P values were calculated using  $\chi^2$  test for discrete variables and two-paired t-test for continuous variables.

P<0.05 was considered as significant

## **2.6 Difference in TL of non-calcified areas between AS patients and control group**

To examine potential TL differences between AS and Non-AS groups, independently of the presence of local calcifications, we compared data from non-calcified valve areas of these 2 groups. We found that patients with AS had significantly shorter telomeres than those from the Non-AS group (8.40±0.64 kb versus 8.85±0.65 kb, p=0.01; Figure 27A). After adjustment for age and sex, this association became even stronger (p=0.004). The mean gap in TL between AS and Non-AS patients was 450 bases. In addition, no difference was observed between TL in Non-AS non-sclerotic and Non-AS sclerotic patient groups (Figure 27B).



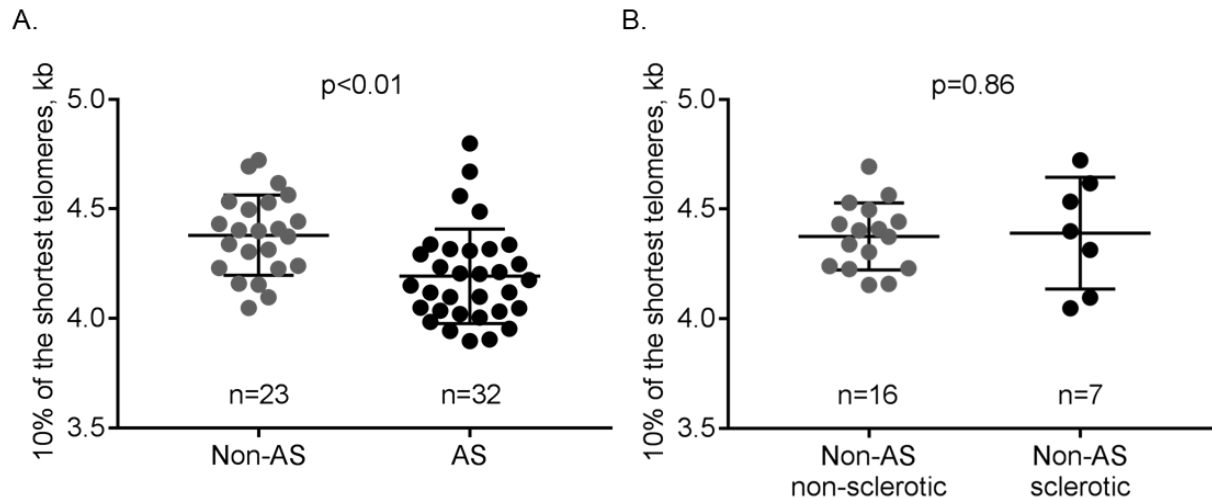
**Figure 27. Telomere length in non-calcified valve areas of patient groups**

Telomere length in Non-AS and AS patient groups (A). Telomere length in Non-AS non sclerotic and Non-AS sclerotic patient groups (B). Data are presented as mean±SD. Statistical analysis was performed using two-sample t-test.

AS = aortic stenosis.

## 2.7 The shortest telomeres in non-calcified areas of AS and Non-AS patients

We also computed mean TL value of the shortest 10% of telomeres in the whole distribution to evaluate the load of short telomeres in aortic valve areas. Briefly, the higher is the load of short telomeres the smaller is this value. Thus, significantly lower mean TL value of the shortest 10% of telomeres in non-calcified valve areas of AS patients than those of Non-AS patients corresponds to higher short telomere load in AS patients ( $p < 0.01$ ; Figure 28A). Additional comparison of mean TL value of the shortest 10% of telomeres in Non-AS non-sclerotic and Non-AS sclerotic patients revealed no difference between those groups (Figure 28B).



**Figure 28. Telomere length in 10% of the shortest telomeres of patient groups**

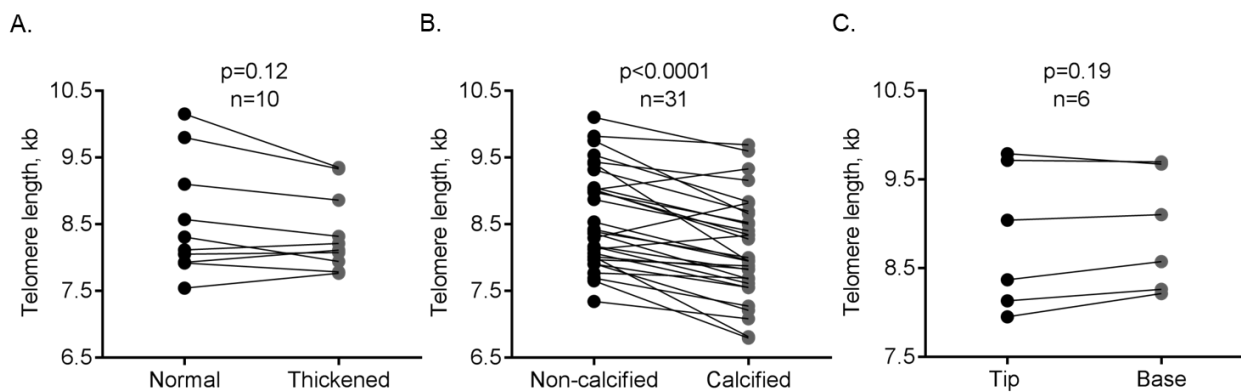
Mean TL value of 10% of the shortest telomeres in Non-AS and AS patient groups (A). Mean TL value of 10% of the shortest telomeres inof Non-AS non sclerotic and Non-AS sclerotic patient groups (B). Data are presented as mean±SD. Statistical analysis was performed using two-sample t-test.

AS = aortic stenosis.

**2.8 TL in different tissue categories of calcified valves**

Normal, thickened and calcified areas of aortic valve represent different stages of AS development [47]. Thus, to identify possible changes in TL with disease progression, we first compared TL in normal and thickened valve areas of the same subjects (n=10). No difference between TL in these two categories of tissue was observed (8.55±0.87 kb versus 8.38±0.60 kb, p=0.12; Figure 29A). Therefore, normal and thickened areas of valve tissue were merged together and called “non-calcified” areas and served as the control for calcified areas in subsequent analyses. Next, we compared TL in patients having valves with both non-calcified and calcified areas (n=31) and found that calcified areas had significantly shorter TL than non-calcified areas (8.58±0.73 kb versus 8.12±0.75 kb, p<0.0001; Figure 29B). On average, telomeres in calcified areas were 462 bases shorter than in non-calcified areas.

In addition, to evaluate telomere dynamics in the absence of macroscopic calcification we measured TL in valve areas obtained from patients who had undergone aortic valve replacement surgery due to aortic valve regurgitation and had non-stenotic valves (n=6). TL was measured in two anatomical areas: tip and base of the valve. No difference between TL in these two anatomical areas was observed (tip: 8.83±0.80 kb versus base: 8.92±0.67, p=0.19, Figure 29C).

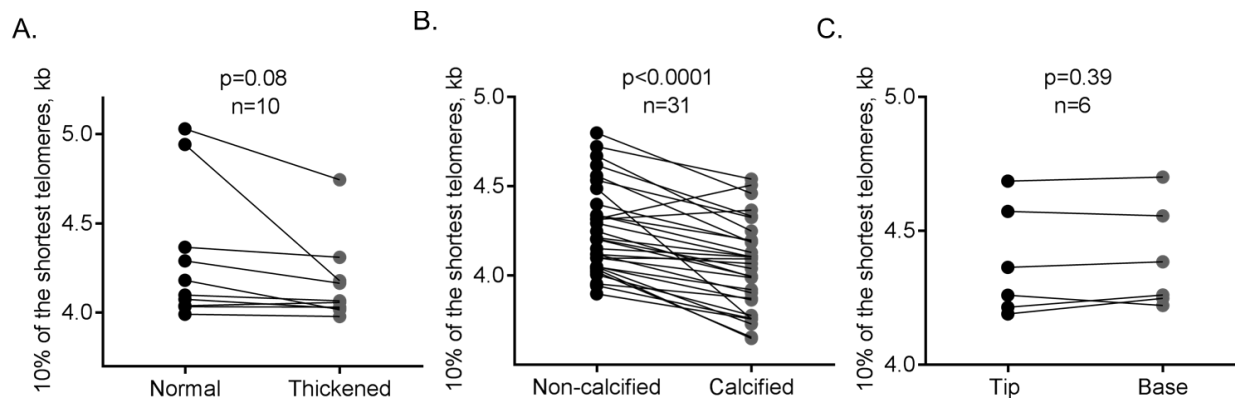


**Figure 29. Telomere length in different categories of the valve tissue**

Telomere length in normal and thickened valve areas (A). Telomere length in non-calcified and calcified valve areas (B). Telomere length in anatomical areas of the valve (C). Matched circles stands for same subjects. Statistical analysis was performed using paired t-test.

## 2.9 The shortest telomeres in different tissue categories

Comparison of the mean TL value of the shortest 10% of telomeres in normal and thickened areas revealed no statistical difference between those tissue categories (Figure 30A). Conversely, mean TL value of the shortest 10% of telomeres was significantly smaller in calcified valve areas than in non-calcified areas, indicating a higher load of short telomeres in calcified areas ( $p < 0.0001$ ; Figure 30B). In addition, comparison of the mean TL value of the shortest 10% of telomeres in anatomical valve areas revealed no differences (Figure 30C).

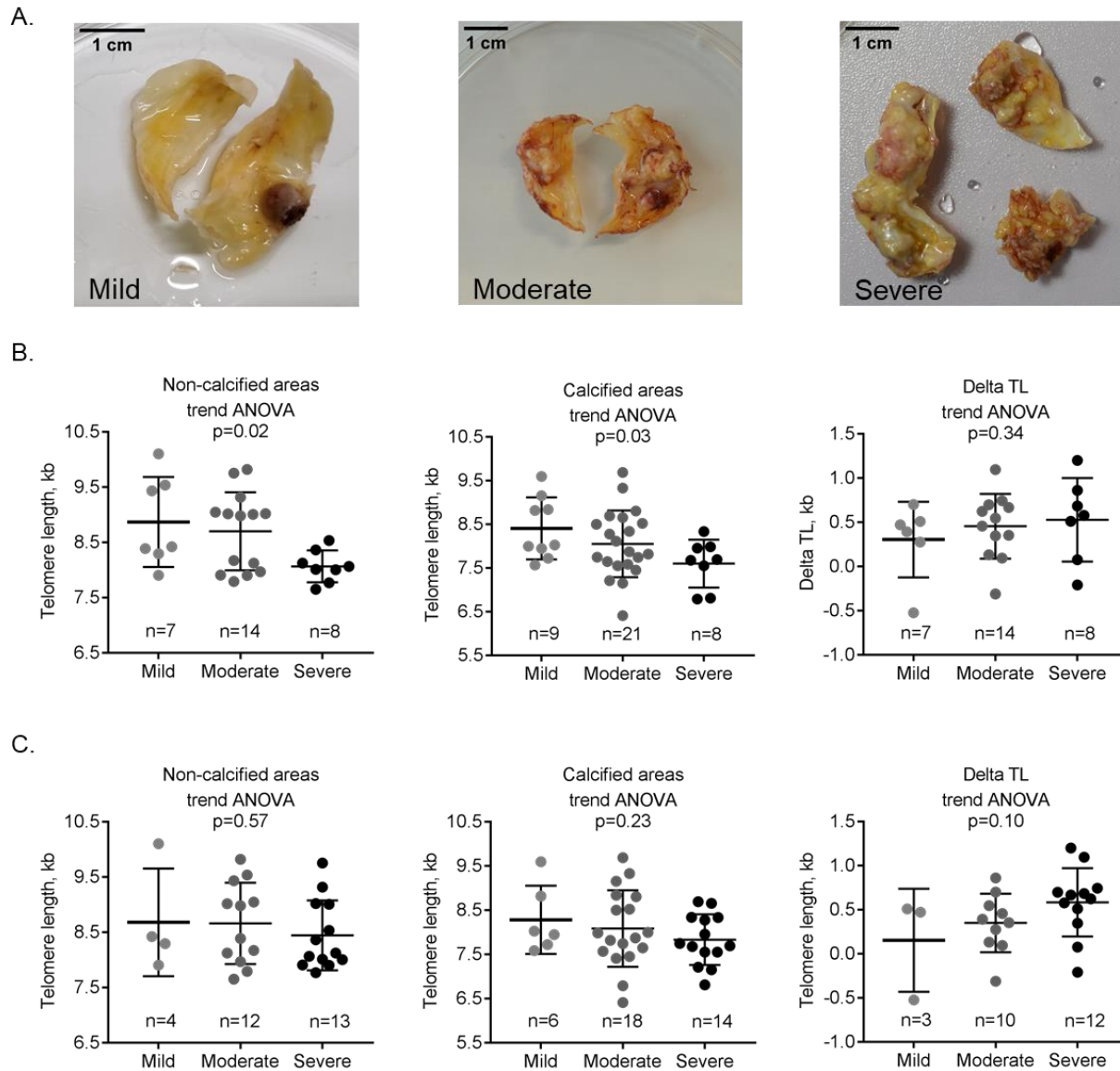


**Figure 30. Telomere length in 10% of the shortest telomeres of different tissue categories**

Mean TL value of 10% of the shortest telomeres in normal and thickened valve areas (A). Mean TL value of 10% of the shortest telomeres in non-calcified and calcified valve areas (B). Mean TL value of 10% of the shortest telomeres in tip and base of the valve (C). Matched circles stand for same subjects. Statistical analysis was performed using paired t-test.

## 2.10 Associations between TL and severity of calcification in valves

We used complete images of whole aortic valves with the presence of macroscopic calcification to scale visually the severity of calcification (Figure 31A). The grouping (mild, moderate or severe) was based on evaluation by two independent observers. The percentage agreement between two observers was 66.7%. Inter-observer variation was moderate (0.43) and intra-observer variation was good (0.71 and 0.73). We compared TL in valves with different degrees of the calcification and observed a decrease in mean TL with higher severity of calcification in both non-calcified and calcified areas of the valves (Figure 31B and C). Conversely, the gap in TL between non-calcified and calcified areas of the same valve (Delta TL) seemed to increase with higher severity of calcification. However, these results were not validated because of discrepancies between the two visual classifications made by the two observers. Significant differences between groups were obtained with classification by observer 1 (Figure 31B) and not reproduced with classification by observer 2 (Figure 31C).



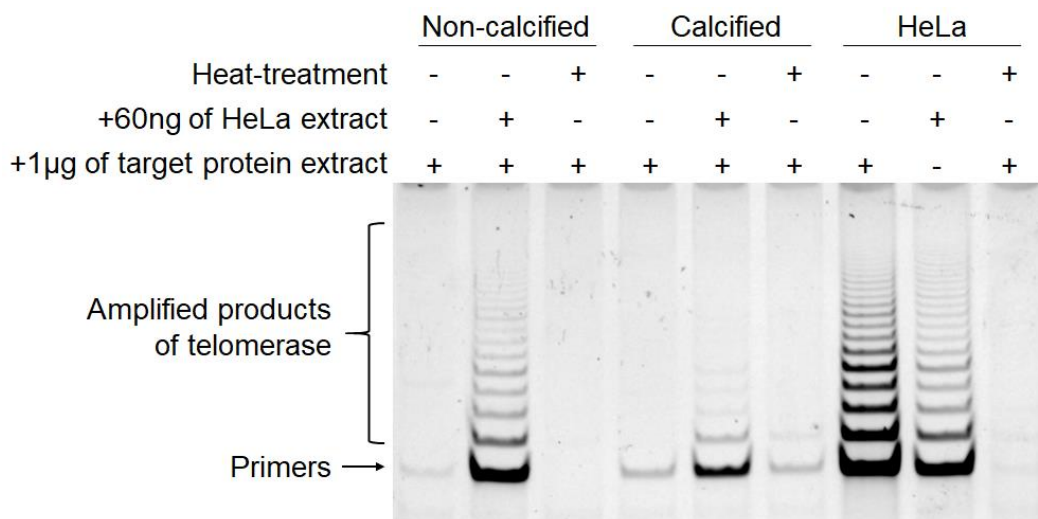
**Figure 31. Association of calcification severity degree and telomere length dynamics in valves with macroscopical calcifications**

Representative images of aortic valves with different degrees of calcifications (A). Telomere length dynamics in stenotic and sclerotic valves with different degree of calcification (scored by observer 1) (B). Telomere length dynamics in stenotic and sclerotic valves with different degree of calcification (scored by observer 2) (C). Delta TL indicates difference between telomere length in non-calcified and calcified areas of the same valve. Data presented as mean $\pm$ SD. Statistical analysis was performed using one-way ANOVA with linear trend analysis



## 2.11 Telomerase activity in the valve tissue

To examine whether telomere maintenance plays a role in valve telomere dynamics, we measured telomerase activity in non-calcified and calcified areas of valve tissue by a modified TRAP assay. Telomerase activity was evaluated in valves obtained from 4 individuals (2 men and 2 women) in the age range 27-70 years. The results were assessed visually for the presence of amplified products of telomerase. Overall, no telomerase activity was detected either in non-calcified or in calcified areas of aortic valve tissue with this approach (Figure 32). Low inhibition of the TRAP assay was observed in reactions which contained protein extract from calcified areas (difference in intensity between lanes 5 and 8, Figure 32).

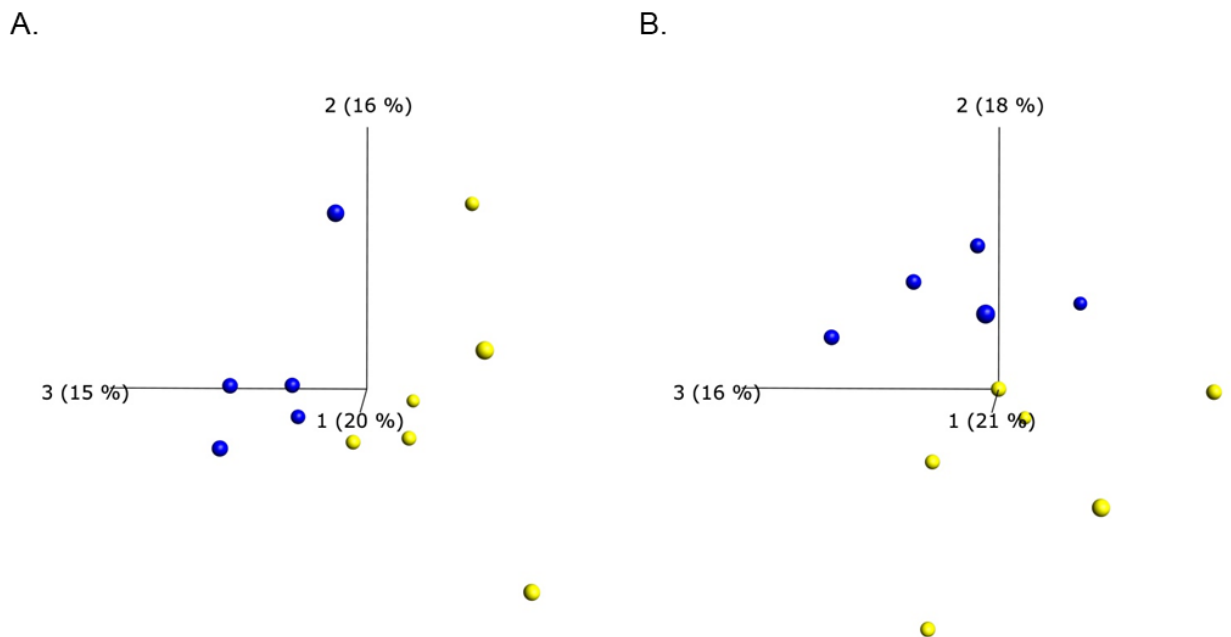


**Figure 32. Representative picture (n=4) of telomerase activity measured in protein extracts from non-calcified and calcified areas of the valve**

Each line corresponds to telomerase activity measured in 1µg of protein extract. Samples, which contain valve tissue extracts mixed with HeLa cell extract, serve as controls for false-negative results and heat-treated samples represent controls for false-positive results. Protein extract from Hela cells was used as positive controls for the reaction.

## 2.12 Association of TL with transcriptomic profile

To explore the potential effect of TL on gene expression profile in valve tissues, we performed a global PCA projection of transcriptomic data obtained in 11 samples (5 non-calcified and 6 calcified areas). These samples were collected from 8 patients, 5 men and 3 women in the age range 63-77 (mean age  $69.5 \pm 5.3$ ). Samples were divided into two groups according to their median TL. Taking into account that different categories of tissue may differ in expression profiles [47], we performed the analysis with and without normalization for the tissue category. Expression profiles from valves with short telomeres clustered together and were distinct from those with long telomeres (Figure 33A). These clusters remained after normalization for tissue category (Figure 33B).



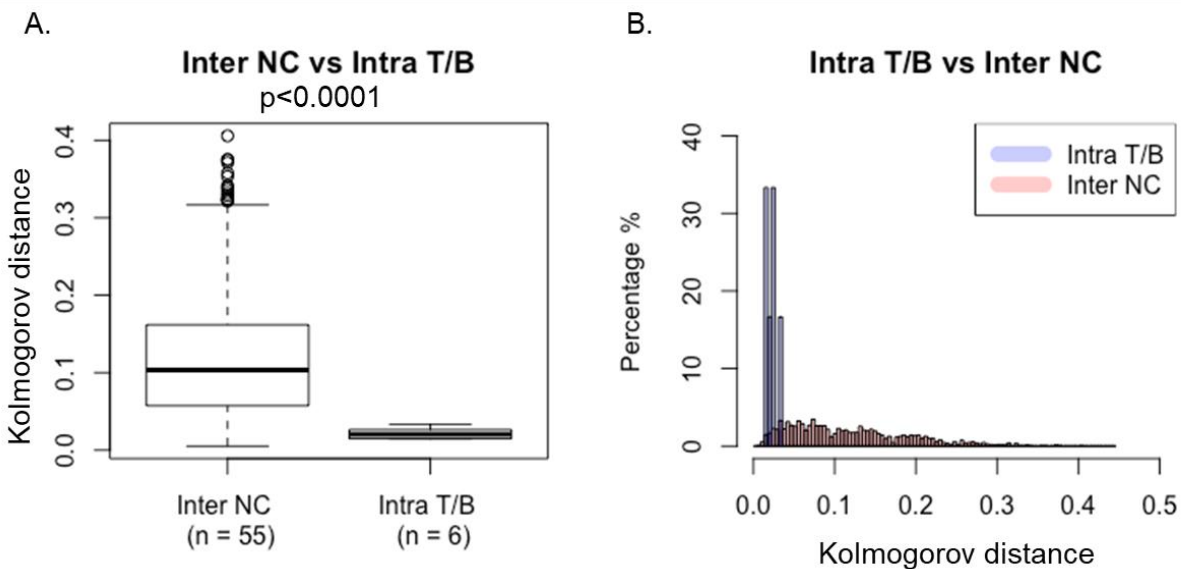
**Figure 33. Principal Component analysis of transcriptome profiles of 11 samples with long and short telomeres**

Analysis performed without normalization for tissue category (A). Analysis performed with normalization for tissue category (B). Yellow dots indicate samples with long telomeres ( $> 7.69$  kb) and blue dots indicate samples with short telomeres ( $\leq 7.69$  kb).

### 3 Changes in TLD shape during development and onset of AS

#### 3.1 Inter-valve and intra-valve TLD shapes in non-calcified areas

Leukocyte TLD shape might be considered as an individual characteristic that is stable over time within individuals and differs between individuals [71]. Currently, nothing is known about changes in valve TLD shape during the pathological process. Thus, we aimed to evaluate changes in valve TLD shape during the development of AS. We used the Kolmogorov distance between TLD to express differences in TLD shape. To achieve our goal, we first analysed intra- and inter-individual TLD in non-calcified areas. Thus, we compared KD between inter-valve TLD in non-calcified areas of the entire cohort and intra-valve TLD in anatomical areas of non-sclerotic valves. We found that intra-valve KD was significantly lower than inter-valve KD ( $p < 0.0001$ ; Figure 34A) indicating that TLD shapes share a lot of similarities within the same valve and differ between individuals (Figure 34B).



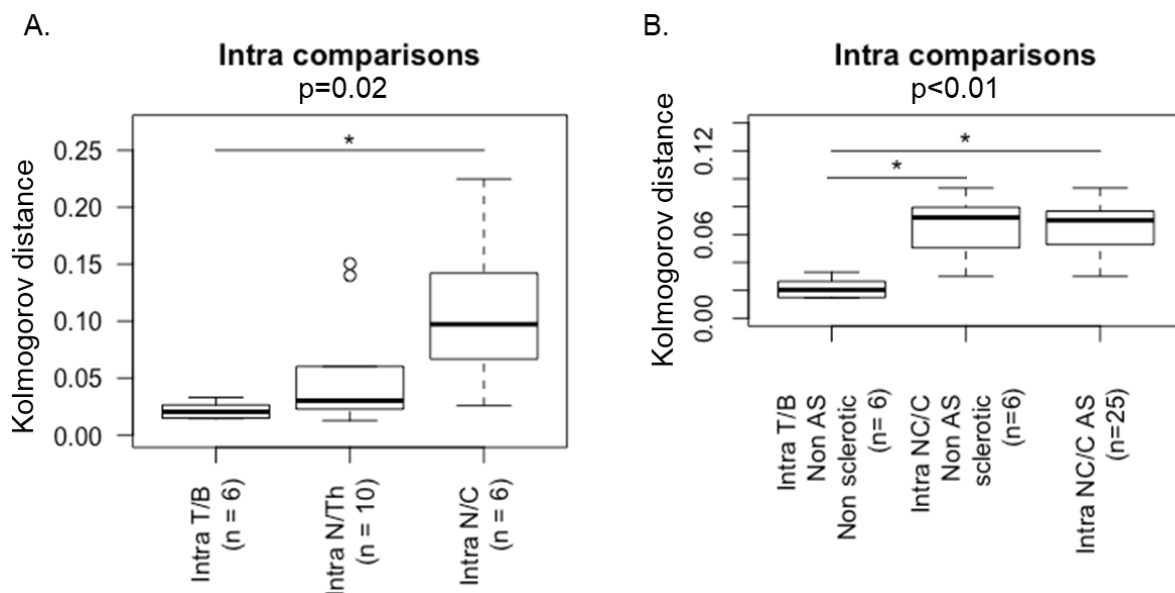
**Figure 34. Intra-valve and inter-valve comparison of Kolmogorov distances between TLD of non-calcified valve areas**

Comparison of intra-valve and inter-valve Kolmogorov distances (A). Intra-valve and inter-valve distribution of Kolmogorov distances between TLD (B). Data presented as median $\pm$ IQR. Statistical analysis was performed using the Mann-Whitney test.

NC = non-calcified, T = tip, B = base.

### 3.2 Intra-valve changes in TLD shapes during development of AS and CAVD

To evaluate changes in valve TLD shape during development of AS, we compared intra-valve KD in non-calcified and calcified valves. Firstly, we assessed changes in TLD shape during CAVD progression by comparison of intra-valve TLD between i) anatomical areas of non-sclerotic valves, ii) normal and thickened areas and iii) normal and calcified areas. We observed gradual changes in TLD shape with progression of CAVD ( $p=0.02$ ; Figure 35A). Secondly, we assessed changes in TLD shape during AS progression by comparison of intra-valve TLD between i) anatomical areas of non-sclerotic valves, ii) non-calcified and calcified areas of sclerotic valves and iii) non-calcified and calcified areas of stenotic valves. We found that variation in shape between calcified and non-calcified areas within both sclerotic and stenotic valves were significantly higher than variation between different areas of non-stenotic valves. No difference was observed in the magnitude of TLD changes between sclerotic and stenotic valves (Figure 35B).



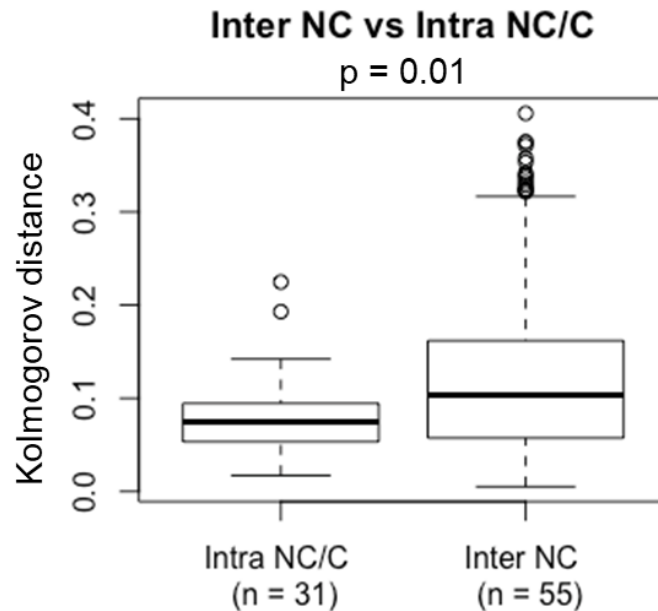
**Figure 35. Intra-valve comparison of Kolmogorov distances**

Comparison of Kolmogorov distances during valve calcification (A). Comparison of Kolmogorov distances during aortic stenosis progression (B). Data presented as median $\pm$ IQR. Statistical analysis was performed using Kruskal-Wallis rank sum test with Dunn test as post-hoc analysis,  $*p<0.01$ .

T = tip, B = base, N = normal, Th = thickened, N = non-calcified, C = calcified, AS = aortic stenosis.

### 3.3 Comparison of intra-valve changes in TLD shapes during calcification and inter-individual variability in TLD shapes in the whole cohort

We further elucidated that changes in TLD shape caused by calcification were significantly lower than inter-individual variation between non-calcified areas in the population ( $p=0.01$ ; Figure 36).



**Figure 36. Comparison of intra-valve Kolmogorov distances between telomere length distribution of non-calcified and calcified areas and inter-valve Kolmogorov distances between telomere length distributions of non-calcified areas**

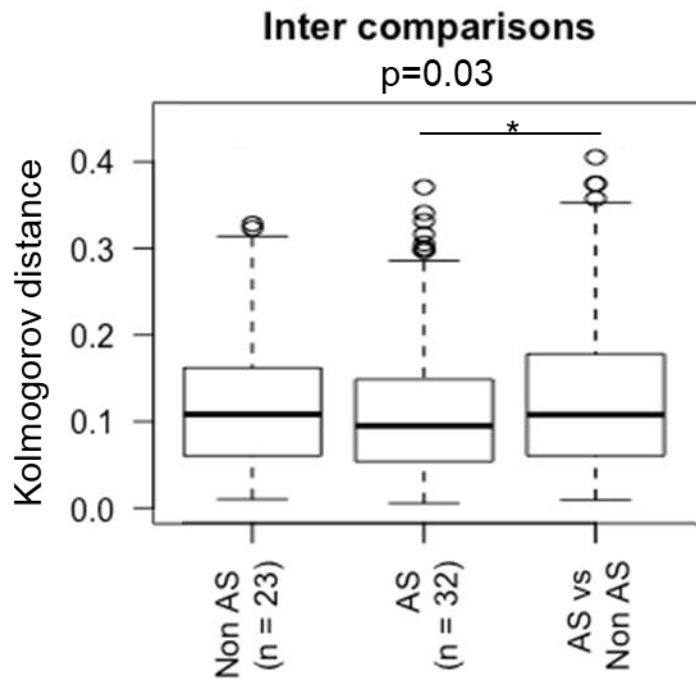
Data presented as median $\pm$ IQR. Statistical analysis was performed using the Mann-Whitney test.

NC = non-calcified, C = calcified.

### 3.4 Comparison of inter-individual variation in TLD shape in non-calcified areas between different groups of patients

Finally, to examine whether there were any similarities in TLD shapes among patients' groups, we compared variabilities in TLD shape in non-calcified areas within the Non-AS group, within the AS group and between Non-AS individuals. Variation was assessed by mean KD between TLD of all the individuals of one group. We observed that variability of

TLD within the AS patients group was lower than variability between Non-AS and AS individuals (Figure 37). This suggests that TLD shape of AS individuals displays some similarities.



**Figure 37. Comparison of inter-valve Kolmogorov distances between telomere length distribution of non-calcified areas of patient groups**

Data presented as median±IQR. Statistical analysis was performed using Kruskal-Wallis rank sum test with Dunn test as post-hoc analysis, \*p<0.01.

AS = aortic stenosis.

# Discussion

In this thesis we studied telomere dynamics in aortic valve tissue in AS and according to the presence of aortic valve calcifications. The four main findings from this work are: 1) patients diagnosed with AS have shorter telomeres in non-calcified areas of their valves in comparison with individuals without AS; 2) valves with CAVD display shorter telomeres in their calcified areas than in non-calcified areas; 3) valve areas with shorter telomeres present distinct transcriptomic profiles from those with longer telomeres; and 4) CAVD and AS have an impact on TL distribution in valves. Taken together, these results suggest that telomeres may play a complex role in disease progression. On the one hand short telomeres in calcified areas seem to be a local consequence of mechanisms involved in the calcification process. On the other hand, short telomeres may participate in the onset and development of AS.

## **1 Telomere dynamics in aortic valve tissues: knowledge from cross-sectional analysis**

In this first study to examine TL directly in valve tissues, we observed a negative association trend between TL in non-calcified areas of valves and age. The calculated cross-sectional rate of TL shortening with age was equal to 15 base pairs per year, which is similar to the telomere attrition in skeletal muscles, a low proliferative tissue [20]. Moreover, TL in non-calcified valve areas of the entire cohort was  $8.59 \pm 0.68$  kb which is equivalent to TL in muscle of same age patients from the TELARTA study (n=259, muscle TL:  $8.54 \pm 0.72$  kb; age:  $63 \pm 14$  years) [20].

We did not observe any association between TL and sex, which may be explained by a relatively small number of women in the study cohort. In addition, TL in valve tissues was independent of the presence of congenital abnormalities, such as a bicuspid valve. Of note, in our study, the proportion of tricuspid valves was higher in the Non-AS group, although, on

average, individuals with bicuspid valves undergo aortic valve replacement surgery with approximately the same frequency as those with tricuspid valves [11]. This difference may have been caused by the selection of the samples. Indeed, tricuspid valves with AS were also used for other ongoing studies at Karolinska institutet. However, since we did not observe any difference in TL between individuals with bicuspid and tricuspid valves, sample selection did not affect our results.

## **2 Role of telomerase activity in telomere length dynamics in valves**

We did not detect any telomerase activity in valve tissues in our analyses. This is not in line with preliminary results from a recent study which showed the presence of telomerase in calcifying VICs [21] or with previous studies on ASCVD, which showed the presence of telomerase in atherosclerotic plaques [22,23]. However, it should be mentioned that these results were based on immunochemistry that shows only the presence of telomerase, but not its activity. Given that there are 22 splice isoforms of human TERT and only one of these isoforms are known to be capable for telomere maintenance [24] and that the vast majority of normal human tissues might express inactive variants of TERT [25], in our study we used the TRAP method that assess telomerase activity reliably.

Interpretation of TRAP results may be difficult, therefore we took time to optimize the TRAP method to be confident in the results of these analyses. Indeed, although the TRAP assay can detect telomerase activity with high sensitivity, it can go along with identification of false-negative and false-positive results. Thus, understanding the emergence of false positives and false negatives is important for correct results interpretation.

False-negative results in the TRAP assay are often a consequence of inhibition of the PCR reaction [387]. In order to detect false-negative results, it is recommended to use an internal control [394]. The internal control band was generated by template TSNT and its reverse primer NT, while the TS primer served as a forward primer [387]. The presence of the



internal control band indicates that the PCR was carried out properly. Disappearance of the internal control band corresponds to inhibition of Taq-polymerase that indicates false-negative results [387]. Of note, intensity of ITAS decreases with an increase of telomerase activity [387]. However, it was previously shown that the internal control with a short primer template, like TSNT, can be amplified in the presence of PCR inhibitors and give false positive results [391]. Therefore, we choose to use another type of control to rule out false negative results. We ran for each sample additional controls, which consisted of protein extracted from HeLa cells mixed with samples of interest before subjecting them to the TRAP reaction. We detected a partial inhibition of the TRAP reaction in the samples extracted from valves, especially in those from calcified areas. This can be due to PCR-inhibitors in protein extracts from valve tissue, especially calcium salts and collagen [395]. Indeed, calcium is very similar by electrochemical properties to magnesium, a cofactor of Taq-polymerase, and acts as a competitive inhibitor that leads to a reduction in the total amount of amplified product [395]. Collagen, which is highly abundant in valve tissue [82], can also inhibit the PCR reaction by binding to the DNA template or by interaction with Taq-polymerase [395].

False-positive results can happen in the TRAP reaction when artifacts mimic the presence of telomerase activity in the absence of active telomerase in the sample. These artifacts can be observed in negative controls which result from amplification of primer-dimer formations [396]. The false-positive results are visually distinguishable from the 6-base-pair ladder, which is the result of genuine telomerase activity [396]. They are characterized by the different signal intensity of the bands and the absence of progression of the signal close to the primer bands [396]. We observed formation of false-positive results in our reactions, which were performed with Taq-polymerase from *Th. aquaticus*. This polymerase lacks 3'→5' proofreading exonuclease activity, which may lead to accumulation of mismatches in the amplified products [397] and, as a result, to formation of primer-dimers due to Taq-polymerase slippage. In addition, the TRAP assay should be less efficient at lower enzyme concentrations or higher annealing temperatures leading to less signal. However, when

using Taq-polymerase from *Th. aquatic* we did not observe a decrease of signal in the 6-pair-base ladder neither in reactions, which contained less HeLa extract nor in reactions with higher annealing temperatures. To rule out false-positive results we used FastStart Taq-polymerase, that display 3'→5' exonuclease activity, and applied additional controls to detect possible primer-dimer formation. This control reaction was assayed alongside each sample and contained heat-inactivated target protein extracts.

With these optimizations and new controls, we have confidence in our results that indicate the absence of telomerase activity in valve samples with the limit of detection we defined (equivalent to 60ng of HeLa protein extract). Our results are consistent with other studies in humans, which show that telomerase activity is repressed in most cells [239], except germline cells [239], cancer cells [240–243], stem cells [244] and activated lymphocytes [245]. On the other hand, we do not deny the potential presence of inactive telomerase isoforms in calcified aortic valves, as they could be involved in noncanonical pathways [26].

### **3 Short telomeres in calcified areas of CAVD valves as a consequence of the calcification process**

In valves with CAVD, we found that calcified areas displayed shorter telomeres than non-calcified areas. This difference could either stem from a direct effect of CAVD or its presence in valves before the disease.

To investigate the temporal relation between short telomeres and calcification, we evaluated TL in anatomical areas of valves macroscopically free of calcification. It was assumed that calcified nodules initially would appear at the sites of increased hemodynamic and biomechanical forces acting on the valve [27], namely the base of the leaflet, and then propagate toward the tip [28]. The lack of difference in TL between these areas supports the idea that short telomeres in calcified aortic valve lesions might be an after effect of the calcification process. Of note, these findings are in contradiction with previous studies,

which show that vascular tissues exposed to the greater hemodynamic stress have shorter telomeres than those with less shear stress [29,30]. However, little is known about initial TL in these different regions of the vascular tree, as well as about the effect of hemodynamic stress on telomere attrition.

The mean gap observed between non-calcified and calcified areas of valves was 462 bases. A rough calculation based on the estimation of valve tissue attrition rate suggests that the difference in TL between calcified and non-calcified areas is equivalent to at least 30 years of “age effect”. If we assume that short telomeres observed in calcified areas are a consequence of the calcification process, it can originate from either local telomere attrition and/or infiltration of immune cells.

Local telomere attrition might be caused by massive oxidative stress and chronic inflammation [31]. Indeed, calcified aortic valve lesions have decreased antioxidant capacity, increased ROS generation as well as increased infiltration of immune cells [32–36]. Oxidative stress and chronic inflammation have been shown to cause telomere attrition *in vitro* [37,38] and *in vivo* [39–41] through ROS generation. ROS are known to induce oxidation and DNA breaks in telomere sequences much more frequently than in other DNA sequences [42,43]. In addition, ROS have multiple effects on biological systems, such as DNA oxidation, that may cause indirect influence on telomere maintenance [37,44], and lead to impaired repair and telomere loss. Moreover, our results are in accordance with previous studies on human vascular tissues reporting that atherosclerotic plaques, where development is associated with chronic inflammation and oxidative stress, have shorter telomeres than healthy vessels [29,41,45].

In our study, further support for local TL attrition by oxidative stress comes from the lack of difference in TL between normal and thickened areas of valves. Although thickening of aortic valve tissue is one of the pathological stages that precede calcification [46], the thickened areas are characterized by increased expression of superoxide dismutase 3 [47]. This antioxidant enzyme may prevent oxidized DNA damage in VICs [36] and thus hamper

telomere loss in thickened valve areas. Telomere attrition would then occur during the next stages of calcification that are characterised by increased oxidative stress and inflammation [28,32,35] .

Besides oxidative stress, chronic inflammation results in abundant infiltration of immune cells in the lesions [28,32]. Thus, calcified areas, in addition to resident cells, comprise inflammatory infiltrates [28,32]. Therefore, TL measured in bulk aortic valve areas reflects their cellular composition. Taking into account previous studies on human tissues, which showed that TL in blood cells is shorter than in other somatic tissues [20,48,49], we assumed that immune cells may have shorter telomeres than resident cells of the aortic valve. Hence, abundant immune cell infiltration in calcified areas of valves could partially contribute to the observed shorter telomeres. In support of this, we observed no difference between TL in normal and thickened areas of the valves, which are less infiltrated with immune cells in comparison with calcified areas [28,32].

#### **4 Short TL precedes AS and may participate in the onset and development of CAVD**

In this study, we found that patients with AS displayed shorter telomeres in non-calcified areas of the valve than those of subjects without AS. This finding provides a suggestion that short telomeres are present in these individuals independently of disease development. We assume that short telomeres may precede calcification of the valve and therefore contribute to disease onset and progression. Indeed, short telomeres have been proposed to be not only a major biomarker of biological aging [50] but also a primary cause of it [14]. Alterations associated with short telomeres in cells include replicative senescence [14]. In turn, senescent cells are suggested to accumulate with age and promote age-related changes in tissues throughout paracrine regulation of neighbour cells by their secretory phenotype [14,15]. Factors released by senescent cells increase chronic inflammation, remodel ECM, impact regenerative capacity and thus contribute to functional changes in tissues and development of age-related diseases [14,15].

Before triggering replicative senescence, telomeres have been also shown to alter gene expression by TPE and TPE over long distances [51]. An increasing number of molecular studies demonstrated the importance of TL in regulation of gene expression [16,52–54]. For instance, using cells with different TL, Lou and colleagues verified the impact of TL on interferon-stimulated gene 15 (*ISG15*) expression in human fibroblast [55]. In this study, telomere shortening lead to up-regulation of *ISG15* [55], which encodes ISG15, an ubiquitin-like protein that is a component in the innate immune response pathway. The author suggested that ISG15 may contribute to age-related inflammation and thus be associated with cardiovascular diseases [55]. Subsequent studies revealed a spectrum of genes, whose expression is regulated by TL [16,52–54]. Among them genes were found that encode proteins, which might be potentially involved in aortic valve calcification, namely osteogenic morphogen bone morphogenetic protein 6 and forkhead box C1 [16,19,56]. Moreover, a recent study in mice showed that genes dysregulated in calcified valves are likely to be regulated in a TL-dependent manner [18]. In our preliminary study, we used transcriptomic analysis to demonstrate that regardless of the calcification status, the expression profile differs between valve areas with long and short telomeres. Thus, we assume that TL by itself may partially contribute to AS development through regulation of gene expression. Of note, these results are preliminary data based on a limited number of samples and should be completed with more samples. This will allow us to provide further details on the genes driving the first principal components and elaborate a pathway for the role of telomere length in AS development through regulation of gene expression.

## **5 Possible involvement of TL in other cells in AS development**

Non-resident valve cells may participate in the link between short TL and AS. Indeed, it was shown previously that intra-individual TL across somatic tissues were comparable [48,57,58]. This actually means that subjects with short telomeres (relative to their peers) in one somatic tissue also usually display relatively short telomeres in other somatic tissues

[48,57,58]. Hence, we may assume that patients with AS have short telomeres in both valves and leukocytes. This assumption is supported by the study of Kurz et al [59], where shorter LTL were shown to be associated with AS. Recently, a new hypothesis to explain short LTL implication in atherogenesis was proposed [60]. Individuals with short LTL have a higher prevalence of clonal haematopoiesis [61]. Clonal haematopoiesis occurs when single hematopoietic stem cell lineages undergo clonal expansion and disproportionately contribute to peripheral blood cells. It increases gradually with aging and is associated with a higher risk of ASCVD and upregulation of pro-inflammatory pathways [62]. Expanded T cell clones have been found infiltrated in aortic valve lesions [63,64]. Moreover, clonal haematopoiesis is driven by somatic mutations in recurrent genes in hematopoietic stem cells [65,66] and some of these mutations, particularly in *TET2* and *DNMT3A* genes, have been linked to increased mortality in patients with AS undergoing transcatheter aortic valve implantation [67]. It was suggested that the presence of these mutations contribute to increased secretion of pro-inflammatory cytokines in peripheral blood cells such as T cells and monocytes [67].

An alternative mechanism to explain the association between short TL and AS would be that shorter telomeres in peripheral blood and/or in valve tissue might also reflect shorter telomeres in endothelial progenitor cells (EPC), which display an endothelial repair capacity. In turn, the regenerative capacity of these cells may be reduced in cells with shorter telomeres [68]. Indeed, recent results from our team show that short LTL are associated with short EPC-TL and a reduced number of self-renewing EPC (unpublished data). Diminished endothelial repair capacity of EPC could play a role in impaired endothelial lesion healing in the first step of AS [27,69]. Thus, shorter telomeres in leukocytes, endothelial progenitors and in valves might play a complex role in AS development and progression. Unfortunately, we did not get access to leukocytes of our cohort that could have allowed us to verify this assumption.

## **6 Role of the shortest telomeres in disease progression and methodological approaches**

In a cell, shortening to a critical length of a small subset of telomeres is sufficient to lead to the onset of replicative arrest [70], which in turn leads to the entire spectrum of cellular changes and contributes to the development of age-related diseases. Therefore, in this work we focused not only on mean TL but also on the TL of the shortest telomere of the distribution. We found differences between the shortest telomeres in non-calcified and calcified tissues and between those of AS and non-AS subjects while no differences were observed between non-calcified tissues, namely i) anatomical areas and ii) normal and thickened valve areas. These results reflect our findings obtained with mean TL, lending further credence to the implication of biological mechanisms linked to short telomeres in AS development.

## **7 TLD in valve tissue and changes in TLD during development of AS and CAVD**

Analyses of whole telomere length distributions in tissue and their changes in shape could provide additional information about the telomere dynamics during disease progression. It was previously shown in leukocytes that even people with the same mean TL may have different TLD shapes, which means that they display different proportions of long, medium and short telomeres [71]. It was also shown that the TLD shape in leukocytes is stable over time [71] but little to nothing is known about TLD in tissue. Here, we showed that TLD shape is very similar in different areas of non-sclerotic valves, while between individuals of the studied cohort TLD shape varied a lot. This is close to the TLD signature concept in leukocytes (stability of shape in time) except that this “tissue signature” is a stability of TLD shape in space (different areas of the same tissue). We observed a progressive loss of this “tissue signature” in valve TLD shape during the calcification process. We suggest that the changes in valve TLD may be caused by immune cell infiltration and/or oxidative stress. Indeed, infiltration in calcified areas of cells with probably shorter TL [20,48,49], and

therefore shifted towards short telomeres TLD, will cause a change in TLD shape resulting in the combined TLD. In addition, using TRF, it was shown that ionizing radiation-induced oxidative stress can change distribution of long, medium and short telomeres in human umbilical vein endothelial cells [72]. We found changes in TLD shape in calcified areas of valves from patients with aortic sclerosis and aortic stenosis, however, the magnitude of the changes was similar in those two groups. We suggest this could be explained by the more prominent inflammation during early stages of CAVD development in comparison with end stages, which are characterized by accelerated calcification [73,74]. Therefore, established changes of TLD shape in patients with aortic sclerosis might hold in AS patients.

Interestingly, although we observed intra-valve changes in TLD shape during AS development, those changes were still smaller than the inter-individual variations of TLD shape in the entire cohort. Further analysis of inter-individual variation of TLD shape revealed that individuals with AS might display more similarities in TLD shape in non-calcified areas between them than with Non-AS patients. If these results were to be confirmed, it will be a first suggestion of a characteristic TLD for individuals more prone to develop AS. However, given the low difference in median values, the high number of observations and the limited significant statistical test, this result should be confirmed by another computational approach. If we assume that there are no differences between variabilities of TLD shapes between the groups, it is an indication that the predisposition to develop AS is not linked to a particular shape/repartition of the TLD but rather to the presence of short telomeres. Of note, when Kolmogorov distances for inter-valve comparison are computed, the number of obtained distances is equal to  $n*(n-1)/2$ , where  $n$  is the number of samples in the group. Therefore, the appearance of outliers may affect the statistical power.

To sum up, using new computational approaches to assess TLD shapes could expand our understanding of telomere dynamics and open new possibilities of interpretation of its links with pathological processes such as valve calcification.



## 8 Study limitations

A first limitation of the present study is the modest number of patients to capture some effects on telomere dynamics with a cross-sectional study. The small number of patients may explain the absence of strong association of TL in non-calcified areas with age, despite the presence of a clear trend to that effect, or with sex. This disadvantage was partly compensated for by using the highly reproducible TRF method for TL measurements. Secondly, we observed a low level of inhibition of telomerase activity assay by protein extracts from valve tissues, which may reduce the sensitivity of telomerase detection and then did not allow us to capture telomerase activity and its variation in tissue. Third, in this study we unfortunately did not get access to leukocytes that could have allowed us to compare telomere dynamics in valves with the more documented telomere dynamics in leukocytes. Fourth, although we suggested that inflammatory infiltrate and oxidative stress may cause the decrease in telomere length in calcified areas, we did not perform specific assessment of these mechanisms in our samples due to the lack of tissue remaining after DNA extraction. Fifth, this study was not designed initially to assess severity of calcification using images of valves. Therefore, exposition and angle of pictures were not standardized, and some images are missing, making the application of any computational tools for automated image analysis impossible. In addition, visual evaluation of the calcification that we used instead is a subjective method and we observed discrepancies between scores of the two observers, making interpretation of these results uncertain. Finally, we did not succeed in establishing cell culture models to allow performing of mechanistic studies of telomere dynamics and their implication in the calcification process.

## General conclusions

In this thesis we explored the implication of telomere dynamics in aortic valve tissue in AS and according to the presence of aortic valve calcifications. Our findings suggest that expanding knowledge in telomere biology could provide a clue to a better understanding of the pathogenesis of cardiovascular diseases. Here, we observed a bilateral relation between TL and progression of degenerative AS. On the one hand, our results suggest that short telomeres might be involved in the onset and development of AS. On the other hand, a local decrease in TL, as well as changes in TLD shape in calcified valve areas may be caused by mechanisms involved in the calcification process.

In this context, future studies should pay more attention to the molecular aspects of telomere dynamics and their involvement in the pathogenesis of AS.

# Perspectives

To go further in the comprehension of the molecular aspects of the relation between TL and AS progression, cellular models are required. These models need to provide support to answer two questions:

## 1 Is calcification causing telomere attrition (*in vitro* model)?

The challenge of this first model is that proliferating cells are needed to answer this question. Accelerated telomere attrition in cell culture is often a result of incomplete replication of chromosome ends due to accumulation of single-strand breaks at telomeres, which are associated with increased oxidative stress [337,398]. Thus, to observe telomere shortening resulting from telomere damage, it is important that the cells divide under the experimental conditions. On the other hand, VICs consist of phenotypically and functionally distinct subpopulation of cells [154], with possibly different proliferation histories. During the cell culture and passages, VICs undergo clonal succession and a relative proportion of subpopulations change: individual subpopulations overgrow previously dominant ones [154]. Thus, an *in vitro* study of VICs should be used on early passages [100,154]. Finally, the method selected for TL measurements in this model should be sensitive enough to detect attrition.

In this study, we used the highly reproducible TRF method to measure TL. However, although TRF is considered to be the gold standard for TL measurements, its measurement error should be taken into account when setting up an experimental design [379]. In our study this error was equal to 2.07%, which amounts to 175 nt for a mean value of 8.5 kb. Hence, to detect any difference in TL between treated cells and control cells, the observed difference should be higher than the measurement error. In addition, the TRF method requires at least 1 µg of DNA (3µg at best), which is hard to obtain from primary cell cultures on early passages. Unfortunately, our attempts to establish a cell culture model that

combines cell proliferation, cell expansion without high number of passages, in procalcifying or osteogenic conditions haven't been successful (results are not shown). Thus, we suggest that in future studies it would be of interest to compare not telomere attrition but telomere damage in cells treated with procalcifying/osteogenic medium and in control cells. This would be possible to achieve by using telomere dysfunction induced foci analysis, which is based on co-localization of markers of DNA damage and shelterin proteins [399]. This technique would allow using less cells, keeping them at low passages to maintain their phenotypes.

## **2 Are short telomeres causing calcification (*in vitro* model)?**

To study implication of short telomeres in the calcification process, it would be of interest to compare the response of VICs with different TL to induction of calcification in cell cultures. Partially, it is possible to achieve this by using cells derived from valves of patients with different TL. TL is highly variable between individuals, and thus it is variable between primary cells obtained from different donors. Indeed, it is also known, that cells derived from different subjects have different calcification potential [400]. However, it should be taken into account that in such an experiment it would be hard to interpret the results due to other differences in genetics between these cells. Another solution would be to test different passages of the same cells to allow telomere attrition during proliferation. But again, changes in VICs with passages cannot be limited to telomere loss as stated before.

The other possible approach is to create isogenic cell lines with different TL. The first study on such kind of cells was performed in 2013 by Stadler G. and colleagues, who created an isogenic cell line with different TL by reversible immortalization of primary cells. Briefly, cells were transduced with a plasmid that coded TERT flanked by two loxP sites, which contained the sequence that was recognized and cleaved by Cre-recombinase. This property of the plasmid was used to excise TERT by transfection of the cells at different time points with another plasmid encoding Cre-recombinase. Thus, obtained isogenic clones had only

significantly different TL due to different periods of telomerase activity. These cell lines were used to investigate regulation of gene expression by different TL [52]. We assume that it will be also interesting to use a similar approach to examine implication of TL in the calcification process. However, we cannot exclude that such transformation of cells will change the phenotype of VICs. To conclude, establishment of experimental conditions to study the influence of telomeres with different lengths on cellular processes is a big challenge.

Aside from the cellular models, this work also opens up further perspectives.

### **3 Study of the potential role of TL in calcification severity**

It would be of interest to examine whether there is a relation between the severity of aortic valve calcification and TL. To this end we suggest the use of two parameters - percentage of the calcified valve area [401] and weight of the valve [32] - to assess the severity of aortic valve calcification, as well as a relatively large sample size (at least 10 valves in each severity group) for the comparison. In addition, having leukocytes for LTL measurements for such a kind of study would also be an advantage.

### **4 Potential impact of TL on genes regulation**

The elucidation of the potential role of telomeres in gene expression promises novel insights into the involvement of TL in age-related diseases. Future research efforts should include more complex transcriptomic analyses with larger sample sizes and identification of candidate genes that are differently expressed in valve tissue with long and short telomeres, potentially linking them to the calcification pathways. Additional studies of gene expression in VICs, applying techniques that provide information about spatial organization of the chromatin, such as Hi-C [402], will allow identification of telomere-contacting promoters, and thus genes, which could be potentially regulated by telomeres. This approach should

provide new gene candidates for further investigations of the role of telomeres in regulation of gene expression in AS.

## **5 Clinical perspectives**

This study has also more distant ramifications and perspectives with possible application in patient care. If the future mechanistic studies tend to confirm the causal role of short TL in valve in AS development, additional studies to elucidate whether individuals with short telomeres in valves also display short telomeres in leukocytes will be needed. This will potentially allow identification of individuals at high risk for aortic valve stenosis in clinical and laboratory practice due to easy accessibility of leukocytes. Control for modifiable cardiovascular risk factors or management of short TL-induced pathways could be proposed to these individuals before the onset of aortic stenosis.

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## Abstract

Aortic stenosis (AS), an age-related disease characterized by narrowing and obstruction of the aortic valve opening, is often related to progressive valve calcification. Mechanisms of valvular calcification involve several pathways influenced by aging. Some of these pathways might be induced by short telomeres, one of the hallmarks of aging. Currently, however, the role of telomere length (TL) in the onset and progression of AS remains unclear.

To shed light on the implication of telomere dynamics in aortic valve calcification and aortic stenosis, we measured TL in aortic valves from patients with and without AS. We found that non-calcified areas of stenotic valves display shorter telomeres than corresponding valve areas of non-stenotic valves. Furthermore, we observed that within a calcified valve, calcified areas exhibit shorter telomeres than non-calcified areas, whereas telomerase activity was not detected in any of those. In addition, study of TL distributions (TLD) showed that, beside mean TL, calcification is associated with changes in TLD shape. Finally, preliminary results of a transcriptomic analysis revealed that TL in aortic valves might be involved in the regulation of gene expression.

These results suggest that short telomeres in aortic valves may contribute to the onset and development of AS. In addition, a further decrease of TL in calcified areas seems to be an after effect of the calcification process. These findings may have long-term ramifications that will potentially allow identification of individuals at high risk for aortic valve stenosis during the aging process. For a better understanding of the involvement of telomere length in aortic valve calcification further mechanistic studies are required.

**Key words:** telomere dynamics, telomerase activity, aortic valve, aortic stenosis, calcification, aging.

## Résumé

La sténose (ou rétrécissement) aortique (SA), une maladie dégénérative liée à l'âge et caractérisée par une diminution de la surface d'ouverture de la valve aortique, est souvent liée à une calcification progressive de la valve. La calcification valvulaire met en jeu des mécanismes impliqués dans le vieillissement, dont certains pourraient être induits par des télomères courts, l'un des mécanismes majeurs du vieillissement cellulaire. A ce jour, le rôle de la longueur des télomères (LT) dans l'apparition et la progression de la SA reste incertain.

Afin d'étudier l'implication de la dynamique des télomères dans le processus de calcification des valves aortiques, nous avons mesuré la LT dans des valves aortiques issues de patients avec et sans SA. Nous avons constaté que les zones non calcifiées des valves sténosées présentent des télomères plus courts que celles des valves sans sténose. De plus, nous avons observé qu'au sein d'une valve calcifiée, les zones calcifiées présentent des télomères plus courts que les zones non calcifiées alors que l'activité de la télomérase n'a été détectée dans aucune de celles-ci. Par ailleurs, l'étude des distributions des LT (DLT) a montré qu'au-delà de la LT moyenne, la calcification est associée à des changements de la forme de la DLT. Enfin, les résultats préliminaires d'une analyse transcriptomique ont révélé que la LT dans les valves aortiques pourrait être impliquée dans la régulation de l'expression des gènes.

Ces résultats suggèrent que des télomères courts dans les valves aortiques pourraient contribuer à l'apparition et au développement de la SA. En outre, le processus de calcification semble entraîner localement une diminution supplémentaire de la LT dans les zones calcifiées. Ces résultats pourraient avoir des ramifications à long terme permettant d'identifier les individus à haut risque de sténose aortique au cours du processus de vieillissement. Pour une meilleure compréhension de l'implication de la longueur des télomères dans la calcification, des études mécanistiques supplémentaires sont nécessaires.

**Mots clés:** dynamique des télomères, activité de la télomérase, valve aortique, sténose aortique, calcification, vieillissement.