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Concerted actions by MMPs, ADAMTS and serine proteases during remodeling of the cartilage callus into bone during osseointegration of hip implants

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ABSTRACT

Introduction: Although the number of patients undergoing total hip arthroplasty is constantly on the rise, we only have limited knowledge of the molecular mechanisms necessary for successful osseointegration of implants or the reasons why some fail. Understanding the spatiotemporal characteristics of signaling pathways involved in bone healing of implants is therefore of particular importance for our ability to identify factors causing implants to fail. The current study investigated the role of three families of proteases, i.e. MMPs (matrix metalloproteinases), ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) and serine proteases, as well as their endogenous inhibitors during osseointegration of hip implants that have endured two decades of use without clinical or radiological signs of loosening.

Materials and methods: Twenty-four patients that had undergone primary THA due to one-sided osteoarthritis (OA) were monitored during 18 years (Y) with repeated measurements of plasma biomarkers, clinical variables and radiographs. All implants were clinically and radiographically well-fixed throughout the follow-up. Eighty-one healthy donors divided in three gender and age-matched groups and twenty OA patients awaiting THA, served as controls. Plasma was analyzed for MMP-1, -2, -3, -8, -9, -10, -13, -14, tissue inhibitor of metalloproteinase (TIMP)-1, -2, -3, ADAMTS4, ADAMTS5, the serine proteases neutrophil elastase (NE), proteinase 3 (PR3) and their endogenous inhibitors, secretory leucocyte proteinase inhibitor (SLPI), trappin-2/elafin and serpina1 (α-1 antitrypsin). Cartilage turnover was monitored using two markers of cartilage synthesis, type II procollagen and PIICP (procollagen II C-terminal propeptide), and two markers of cartilage degradation, CTX-II (C-terminal telopeptide fragments of type II collagen) and split products of aggrecan (G1-IGD-G2).

Results: MMP-1, MMP-9, ADAMTS4, NE and PR3 were above healthy in presurgery OA patients but returned to the level of healthy within 6 weeks (W) after surgery. MMPs and serine proteases were counter-regulated during this phase by TIMP-1, SLPI and trappin-2/elafin. Type II procollagen, PIICP and CTX-II increased to a peak 6 W after surgery with a gradual return to the level of controls within weeks. Significant increases by MMP-8, MMP-9, ADAMTS4, ADAMTS5, NE, PR3 and the protease inhibitors, TIMP-3 and serpina1, were seen 5 Y after hip arthroplasty paralleled by a sharp increase in the levels of the cartilage degradation markers, CTX-II and G1-IGD-G2. All the above mediators were normalized before 18 Y, except MMP-1 and MMP-9 that remained above healthy at 18 Y. MMP-14 increased immediately after surgery and remained elevated until 5 Y postsurgery before returning to the level of controls at 7 Y.

Conclusion: Notwithstanding temporal differences, the molecular processes of bone repair in arthroplasty patients show great spatial similarities with the classical phases of fracture repair as previously shown in animal models. Cartilagenous callus, produced and remodeled early after hip arthroplasty, is replaced with bone 5 Y to7 Y after surgery by the concerted actions of MMP-8, MMP-9, ADAMTS4, ADAMTS5, NE and PR3, thus suggesting that a complex regulatory cross-talk may exist between different families of proteases during this transitional phase of cartilage degradation. Regulation and fine-tuning of cartilage remodeling by MMPs and ADAMTS is controlled by TIMP-3 whereas serine proteases are regulated by serpina1. Increased MMP-1 and MMP-9 beyond 10Y post-THA support a role during coupled bone remodeling.

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1. Introduction

Total hip arthroplasty is one of the most successful surgical procedures of modern time but despite new biomaterials and significant advances in functionality and durability, implants are still associated with failure. In a previous report (Cassuto et al., 2017) we highlighted the importance of understanding the biomolecular mechanisms regulating successful osseointegration of hip implants for our ability to identify and target the biological reasons underlying prosthesis loosening.

Endochondral bone repair begins early by condensation of mesenchymal stem cells (MSCs) into proliferative chondrocytes that secrete large quantities of cartilage-specific type II procollagen into the ECM which is enzymatically transformed into type II collagen that forms the collagen fibrils of the osteoid (Charni-Ben Tabassi and Garnero, 2007). As the spaces between the fibrils become filled with proteoglycans (primarily aggrecan), the osteoid is transformed into the cartilagenous callus which gradually undergoes mineralization while becoming hypertrophic. Remodeling of the mineralized cartilage is initiated by vascular ingrowth which allows chondroclasts and osteoblasts into the avascular tissue where they degrade the cartilage and replace it with woven bone (DeLise et al., 2000). This remodeling process requires the involvement of several major enzymatic pathways, such as MMPs (Krane and Inada, 2008; Paiva and Granjeiro, 2014), ADAMTS (Kelwick et al., 2015) and serine proteases (Korkmaz et al., 2008; Korkmaz et al., 2010). MMPs are a family of zinc-dependent proteases that are generally released into the ECM in the inactive pro-form which requires additional processing by other MMPs as well as enzymes of other families to become active (Krane and Inada, 2008; Paiva and Granjeiro, 2014). Aside from being able to cleave practically all components of the ECM, MMPs also target a wide variety of other molecules, such as other MMPs and proteinases, membrane bound proteins, proteinase inhibitors, growth factors, cytokines, chemotactic factors, as well as cell-cell and cell-ECM adhesion molecules (Krane and Inada, 2008; Paiva and Granjeiro, 2014). By being able to generate and release active molecules embedded within the ECM, MMPs have been proven to play a substantial role in a wide range of physiological and pathological events (Krane and Inada, 2008; Paiva and Granjeiro, 2014). Due to the inherent potency of MMPs to degrade tissues and to cause extensive damage when uncontained, the enzymes are tightly regulated both intracellularly, via gene transcription and translation, and extracellularly through enzymatic degradation and inhibition, the latter by a four-member group of TIMPs. Another family of proteases important in health, but also in human diseases, is the serine protease family which includes, among others, neutrophil elastase, proteinase 3 and cathepsin G (Korkmaz et al., 2008; Korkmaz et al., 2010). Serine proteases are primarily produced and stored in mature and active form within neutrophil granules and released from neutrophils upon their activation (Korkmaz et al., 2008; Korkmaz et al., 2010). In similarity with MMPs, they are capable of degrading most structural components of the ECM as well as activate or inactivate MMPs and cytokines. By being active upon release, serine proteases are subject to rigorous control by endogenous inhibitors, such as trappin-2/elafin, SLPI and serpina1 (Korkmaz et al., 2008; Korkmaz et al., 2010). ADAMTS are closely related to the MMPs as both families have a zinc ion-dependent metalloproteinase active site and need to be activated upon release (Kelwick et al., 2015). ADAMTS4 (aggrecanase 1) and ADAMTS5 (aggrecanase 2) were included in the current study as they, along with MMPs, are able to cleave aggrecan, the second most prevalent component of the cartilage ECM (Kelwick et al., 2015).

The aim of the current study was to investigate the role of MMPs, ADAMTS, serine proteases and their endogenous inhibitors during osseointegration of uncemented total hip implants that had remained stable during two decades of use.

2. Materials and methods

2.1. Study design and study population

Results of the current study originate from the same population of THA patients and controls as a previous report (Cassuto et al., 2017). The study was approved by the institutional review board and informed consent was obtained from every patient prior to inclusion. This study, with level of evidence II, complies with the STROBE-statement for observational studies (von Elm et al., 2014). (I) Sixty consecutive patients scheduled for primary THA due to osteoarthritis (OA) were enrolled into the study to evaluate different femoral stem designs with clinical parameters, radiography, radiostereometry (RSA) and dual-energy x-absorptiometry (DEXA) (Karrholm et al., 2002; Thien et al., 2012). In addition, venous blood was sampled on each visit. The THA group received either of two uncemented stems, i.e. Epoch®, a low-modulus stem with porous coating and reduced stiffness ($n = 10$) and Anatomic®, a titanium alloy stem with porous coating ($n = 14$), both from Zimmer-Biomet, Warsaw, Indiana, USA. Both stem types were supplied with an additional layer of hydroxyapatite and tricalciumphosphate (HA/TCP) on the porous coating. The Anatomic stems were also coated with pure hydroxyapatite distal to the porous coating. All patients received a cementless porous press-fit cup with HA/TCP coating (Trilogy®, Zimmer-Biomet) on the acetabular side fixed with or without additional screws. All cups were supplied with polyethylene liners gamma-sterilized with 0.025 Gy in inert nitrogen gas. Characteristics, such as wear rates, stem migration pattern, loss of BMD and clinical outcome, were reported for the two stem implants in a 7-year follow-up (Thien et al., 2012). Basic demographic data, comorbidities and medications were registered. Patients with pre-existing hip implant prior to the index implant ($n = 14$), were excluded. Patients who developed clinical (see HHS and pain scores below) and/or radiological signs of OA in the opposite hip joint during the course of the follow-up ($n = 12$), were also excluded. Eight patients with lysis or implant wear prompting revision surgery and two patients with clear-cut lysis, but no revision, were excluded. Patients that developed prosthesis loosening during the course of the follow-up were not included in the current investigation as they proved to be too few and too widely apart with regard to temporal presentation of implant failure and thus not eligible for proper statistical evaluation of biomolecular characteristics. Handling of the latter group has been discussed in a previous report (Cassuto et al., 2017). A total of 24 patients (mean age 58Y, range 40–69; 16 males and 8 females) with one-sided total hip implant that had remained clinically and radiographically stable during a two-decade follow-up, with the contralateral hip not showing clinical or radiographic signs of OA, were included. (II) Twenty OA patients awaiting THA (mean age 58Y, range 34–86; 14 males and 6 females), were included as a control of the stability and validity of biomarkers in pooled plasma. Each biomarker was tested by comparing levels in plasma stored for 18Y (presurgery sample, PR) with fresh plasma from OA patients awaiting THA. (III) Eighty-one healthy subjects were divided into three gender and age-matched subgroups (mean age 58Y, 67Y and 79Y) that served as controls to THA patients over the course of the follow-up (Table 1) with age means being chosen to represent an entry, a midpoint and an exit point. Healthy and OA awaiting THA were recruited between 10Y and 13Y after ending inclusion of THA patients. We did not investigate socioeconomic status. Exclusion criteria in both the THA group, the control group awaiting THA and the healthy groups were selected to minimize the risk of interference with the validity of biomarker results. We excluded subjects in all groups suffering from any kind of malignancy, immune disorder (e.g. HIV), immune-related joint diseases (e.g. rheumatoid arthritis), or diseases affecting the bone (e.g. Paget's disease), kidneys or liver. Individuals on steroids, immunotherapy/chemotherapy or bone-regulating drugs (e.g. bisphosphonates, denosumab, calcitonin, PTH) were excluded. In addition, healthy controls were also excluded if they had a history of bone trauma within a year of inclusion or diseases affecting the joints (e.g. osteoarthritis).

Table 1
Demographic and routine laboratory data for the three groups of healthy subjects and for THA patients at inclusion. Comparisons between the healthy groups were made by one-way repeated measures ANOVA whereas comparison between healthy (mean age 58Y) and the THA group (mean age 58Y) were done by Mann-Whitney *U* test. Data were presented in part in a previous publication (see ref. (Cassuto et al., 2017)).

Subjects	Healthy (n = 27)	Healthy (n = 27)	Healthy (n = 27)	THA at inclusion
Age (Y) mean ± SEM (range)	58 ± 1.7 (40–68)	67 ± 0.6 (61–72)	79 ± 1 (71–88)	58 ± 1.4 (40–69)
Gender (F/M)	11/16	8/19	7/20	8/16
Height (cm)	166 ± 7.6	177 ± 1.5	174 ± 1.5	171 ± 5
Weight (kg)	77 ± 4	87 ± 2	77 ± 3	83 ± 4
BMI (kg/m^2)	25 ± 1	27 ± 1	26 ± 0.6	26 ± 1.1
HB (g/L)	133 ± 7	144 ± 2	145 ± 3	140 ± 4
WBC (× 10^9/L)	6.2 ± 0.4	6.3 ± 0.5	6.5 ± 0.3	6.4 ± 0.3
Platelet count (× 10^9/L)	216 ± 17	205 ± 10	216 ± 10	209 ± 12
S-creatinine (μmol/L)	85 ± 12	90 ± 2	91 ± 5	83 ± 8

2.2. Radiographs

Anteroposterior pelvic radiographs plus anteroposterior and axial radiographs of the THA hip and the contralateral hip were done in the THA group and the OA group awaiting THA. Radiographs in THA patients were taken shortly before surgery (PR), on day one after surgery (PO), 6 weeks (6 W), 3 months (3 M), 6 M, one year (1Y), 2Y, 5Y, 7Y, 10Y, 13Y, 15Y and 18Y after the index operation. Radiographs of the hip included the acetabular and femoral portions of the joint with examination for joint space narrowing, subchondral lucency, marginal osteophytes and subchondral sclerosis. In cases with radiographic signs of OA in the contralateral hip joint, the patient was excluded due to potential interference with biomarkers of the joint representing the index operation.

2.3. Harris hip score (HHS) and pain scores

The HHS score gives a maximum of 100 points. Pain receives 44 points (see below), function 47 points, range of motion 5 points, and deformity 4 points. Function is subdivided into activities of daily living (14 points) and gait (33 points). The higher the HHS, the less dysfunction (Harris, 1969). Pain scores in THA patients were recorded before surgery and during the entire follow-up. HHS was measured at each visit and supplemented with a more detailed questionnaire of hip pain graded as follows: 44 (no pain or negligible pain), 40 (mild pain but no functional disability), 30 (no ADL dysfunction but mild pain in connection with physical activity prompting occasional use of analgesics), 20 (ADL partially limited by moderate pain necessitating analgesics on regular basis), 10 (severe pain with pronounced limitation of ADL and regular use of analgesics), 0 (severe and disabling pain at rest with continuous use of analgesics).

2.4. Blood sampling and analysis of plasma biomarkers

Venous blood was drawn during normal working hours into EDTA tubes from THA patients at PR, PO, 6 W, 3 M, 6 M, 1Y, 2Y, 5Y, 7Y, 10Y, 13Y, 15Y and 18Y post-surgery. PR and PO samples were collected on the day, samples at 6 W, 3 M and 6 M varied ± 3 days and samples taken between 1Y and 18Y varied 5–16 days. Venous blood for cytokine analysis in healthy controls and OA patients awaiting THA was sampled on a single occasion. Blood samples for cytokine analysis were centrifuged at 4 °C and immediately stored at -85 °C until analysis. Plasma biomarkers were analyzed on a high-sensitivity and wide-dynamic range platform from MesoScaleDiagnostics (MSD, Sector Imager 2400®; Rockville, Maryland, USA; for details see www.mesoscale.com). Precoated multi-spot 96-well plates from MSD were used for plasma analysis of MMP-1, MMP-3, MMP-9 (Human MMP 3-plex ultra-sensitive kit, K15034C) and MMP-2, MMP-10 (Human MMP 2-plex ultra-sensitive kit, K15033C). Matched pairs of anti-human antibodies, i.e. capture antibody (CA) and biotinylated detection antibody (DA) labeled with streptavidin SULFO-TAG®, were used for analysis of plasma biomarkers as specified below. Standard curves were created using human recombinant proteins (hRP). Plasma was mounted on uncoated standard plates from MSD (L15XA). MMP-8, CA: monoclonal mouse $IgG2_B$ antibody (Biotechne R&D Systems, cat.no.MAB908), DA: biotinylated antigen affinity-purified polyclonal goat IgG antibody (Biotechne R&D Systems, cat.no.BAF908), hRP (Biotechne R&D Systems, cat.no.908-MP). MMP-13, CA: monoclonal mouse IgG_1 (Biotechne R&D Systems, cat.no.MAB511), DA: biotinylated antigen affinity-purified polyclonal goat IgG antibody (Biotechne R&D Systems, cat.no.BAF511), hRP (Prospecbio cat.no.enz-317). MMP-14/MT1-MMP, CA/DA/hRP: (DuoSet, Biotechne R&D Systems, cat.no.DY918). TIMP-1 (plasma diluted 1:50) CA/DA/hRP: (PeproTech, cat.no.900-K438). TIMP-2 (plasma diluted 1:50), CA: anti-human monoclonal mouse IgG_1 antibody (Biotechne R&D Systems, cat.no.MAB9711), DA: biotinylated polyclonal goat IgG antibody (Biotechne R&D Systems, cat.no.BAF971), hRP (PeproTech, cat.no.410). TIMP-3, CA: monoclonal mouse IgG_1 antibody (Biotechne R&D Systems, cat.no.MAB973), DA: biotinylated mouse IgG_{2A} antibody (Biotechne R&D Systems, cat.no.BAM9731), hRP (Biotechne R&D Systems, cat.no.973-TM). ADAMTS4 (aggrecanase-1): CA/DA/hRP (DuoSet, Biotechne R&D Systems, cat.no.DY4307). ADAMTS5 (aggrecanase-2): CA/DA/hRP (DuoSet, Biotechne R&D Systems, cat.no.DY2198). Aggrecan: CA/DA/hRP (DuoSet, Biotechne R&D Systems, cat.no.DY1220) with capture antibody directed against recombinant human aa20–675 in the interglobular domain G1-IGD-G2 of the aggrecan molecule, as stated by the manufacturer. Type II procollagen: CA/DA/hRP (DuoSet, Biotechne R&D Systems, cat.no.DY7589). Neutrophil elastase (NE): CA/DA/hRP (DuoSet, Biotechne R&D Systems, cat.no.DY9167). Proteinase 3 (PR3): CA/DA/hRP (DuoSet, Biotechne R&D Systems, cat.no.DY6134). Serpina1: CA/DA/hRP (DuoSet, Biotechne R&D Systems, cat.no.DY1268). SLPI: CA/DA/hRP (DuoSet, Biotechne R&D Systems, cat.no.DY1274). Trappin-2/Elafin (measures elafin and its precursor trappin-2) (plasma diluted 1:5), CA: antigen affinity purified goat IgG antibody (Biotechne R&D Systems, cat.no.AF1747), DA: biotinylated antigen affinity-purified polyclonal goat IgG antibody (Biotechne R&D Systems, cat.no.BAF1747), hRP (Biotechne R&D Systems, cat.no.1747-PI). Antibodies were optimized by checkerboard titrations and subsequent control of standard curves. Inter-assay variations were < 5%. PIICP (Nordic Biosite cat.no.EKH4286) and CTX-II (Nordic Biosite cat.no.EKH2012) were supplied as kits containing precoated plates, antibodies and hRP and were analyzed by use of colorimetric ELISA (Epoch®, Biotek, Vermont, USA).

2.4.1. Stability of stored plasma

An important issue when storing blood samples over extended periods of time is the degree of degradation which could have significant impact on the interpretation of data. To ascertain the validity of stored plasma, levels of biomarkers in THA patients taken before surgery and stored for 18 years were compared with levels in fresh plasma from OA patients awaiting THA. Inter-group variability was minimized by mounting plasma from both groups on the same analytical plates. Comparison was also made between preoperative biomarker levels of the current study and another longitudinal hip replacement study (*E-poly*) (18Y vs 7Y pooling, unpublished) showing only minor differences. To safeguard the stability of biomarkers over the course of time, EDTA tubes (-85 °C) were thawed on ice in limited numbers and plasma was aliquoted into cryotubes (120 μl/tube) and stored at -85 °C for short periods of time. Each cryotube was only thawed and used on a single occasion for analysis on 4 different plates (25 μl/well, see www.mesoscale.com) with excessive plasma being discarded. Evaluations at our lab showed no distinguishable changes in biomarker levels after 3 freeze/thaw cycles.

2.5. Statistical analysis

Data are presented as the mean ± SEM. One-way analysis of variance (ANOVA) with post hoc Holm-Śidak test was used to compare biomarkers in the THA group, representing a continuous dependent variable (time), versus the independent categorical groups of healthy subjects. Normality was assessed by Shapiro-Wilk and Kolmogorov-Smirnov tests. Equal variance was tested by the Brown-Forsythe test. Log transformation was used to normalize data when necessary. Comparisons were performed as follows: 1) THA PR vs healthy mean age 58 Y (all biomarkers), 2) THA PO, 3 M or 6 M vs healthy mean age 58Y (all biomarkers), 3) THA representing high activity between 5Y and 13Y vs healthy mean age 67Y and 4) THA 18Y vs healthy mean age 79Y (all biomarkers). One-way repeated measures ANOVA with post hoc Holm-Śidak test was used to analyze differences in demographic, laboratory and cytokine data between the three independent groups of healthy subjects (Table 1). Comparisons of biomarker levels within the THA group were done by one-sample *t*-test.

3. Results

Age-related changes in biomarker levels between the 3 groups of healthy are presented in Table 1. None of the differences between the groups were significant. Comparison of demographic and routine laboratory data between healthy controls (mean age 58 Y) and THA-patients at inclusion were not significant (Table 1). There were no significant differences in plasma levels of study biomarkers taken before surgery (PR) in arthroplasty patients (stored 18Y) relative biomarker levels in fresh plasma from OA patients awaiting THA.

HHS and pain scores are shown in Table 2. HHS increased significantly from PR to 1Y after hip arthroplasty ($p < 0.001$) whereas differences between 1 Y post-THA, on one hand, and 5, 10 and 18 Y were not significant. Pain level decreased from moderate pain requiring analgesics before surgery (score = 20) to no pain (score = 44) at 1Y post-THA ($p < 0.001$). Pain at 5, 10 and 18 Y after arthroplasty was not significantly different from pain at 1Y post-THA.

Figs. 1 to 8 show levels of plasma biomarkers in arthroplasty patients throughout the follow-up and relative to the three groups of gender and age-matched healthy controls. Fig. 9 summarizes the spatial changes and interrelation between biomarkers of the current study during the four main stages of bone fracure repair. In short: The proteases MMP-1, MMP-9, ADAMTS4, ADAMTS5, NE, PR3 and the inhibitors TIMP-1 and SLPI were all above healthy in presurgery OA patients with MMP-1, MMP-9 and TIMP-1 increasing further one day after surgery before decreasing to the level of healthy at 6 W post-THA. Plasma levels of the cartilage turnover markers, type II procollagen, PIICP and CTX-II, reached a peak between 6 W and 3 M after surgery followed by a return to the levels of healthy at 6 M. A sharp increase in the levels of MMP-8, MMP-9, ADAMTS4, ADAMTS5, NE, and PR3 at 5 Y post- THA coincided with a similarly sharp increase by the cartilage degradation markers, CTX-II and aggrecan (bi-products). Increased levels of MMPs, ADAMTs and serine proteases at 5 Y were paralleled by similar increase of the protease inhibitors, TIMP-3 and serpina1. All biomarkers of the study had shifted to the level of healthy at 18 Y after THA, except MMP-1 and MMP-9, which were significantly above healthy while their inhibitor, TIMP-1, was below healthy. MMP-13 embarked on a negative trajectory shortly after surgery interrupted by a sharp correction to the level of healthy at 5 Y. MMP-14 increased immediately after surgery and remained elevated until 5 Y postsurgery before returning to the level of healthy controls at 7 Y. MMP-1, MMP-2 and MMP-3 were suppressed below healthy throughout most of the follow-up until being aligned with levels of healthy at 13 Y or increasing above healthy (MMP-1). MMP-10 did not differ from healthy at any point during the follow-up.

4. Discussion

As we monitor THA patients during the sixth and seventh decades of life, often characterized by major changes in determinants of cartilage an bone metabolism as well as augmented musculoskeletal inflammation (Chung et al., 2019), we will discuss age and inflammation as potential confounding factors prior to elaborating on the role of proteases during osseointegration.

4.1. The impact of age

Neutrophils and macrophages often show age-related changes associated with increased mobilization of MMPs and serine proteases (Chung et al., 2019; Freitas-Rodriguez et al., 2017), also reflected in the current population of healthy showing age-dependent increase in neutrophil-derived MMP-8, NE and trappin-2/elafin (Table 3). Similarly, MMP-1, MMP-3 and their endogenous inhibitors, TIMP-1 and TIMP-2, increased with age in healthy individuals (Table 3), thus being in agreement with previous reports (Freitas-Rodriguez et al., 2017; Bonnema et al., 2007). Notably, out of twenty-two proteases/inhibitors analyzed in the current study, nineteen failed to show significant differences between THA and healthy at 79Y of age (Table 3) thus confirming the overall proximity between the age-related trajectories of the two groups and strongly contradicting that the dramatic increases in MMPs, ADAMTS and serine proteases at 5-7Y post-THA are due to progressing age, not least when none were reflected in the population of gender/age-matched healthy controls.

4.2. Inflammatory cytokines – friend or foe?

Despite dramatic increases in the levels of proinflammatory cytokines (IL-1β and IL-8) (Cassuto et al., 2017) and tissue degradative MMPs, ADAMTS and serine proteases at 5-7Y post-THA, there were no reports of pain or other inflammation-related symptoms by patients in the THA cohort during this phase, nor were there any identifiable signs of lysis or other pathologies on plain radiographs. When considering why this early phase of osseointegration was clinically and radiographically "silent" despite the dramatic molecular processes associated with it, it is relevant to ask whether the glass at that point was half empty or half full as proinflammatory cytokines and tissue proteases are janus-faced by being able to trigger and sustain tissue damage during pathological conditions while at the same time being of critical importance for normal bone repair (Marsell and Einhorn, 2011; Mountziaris and Mikos, 2008). The physiological character of the molecular events at 5Y post-THA is supported by being similar to

Table 2
Harris hip score (HHS) and pain scores in THA patients. ***p < 0.001 1Y post-THA versus preoperative values (=PR). Differences at 5Y, 10Y and 18Y post THA versus 1Y post-THA were not significant. Statistical evaluations were made by Mann-Whitney *U* test. Table previously presented (see ref. (Cassuto et al., 2017)).

HHS	PR	1Y	2Y	3Y	5Y	7Y	10Y	13Y	15Y	18Y
Median	54	94***	97	100	96	98	100	96	100	95
Range	25–75	65–100	55–100	70–100	72–100	51–100	50–100	84–100	86–100	89–100
Pain										
Median	20	44***	44	44	44	44	44	44	44	44
Range	0–30	20–44	10–44	20–44	30–44	30–44	30–44	30–44	40–44	44–44

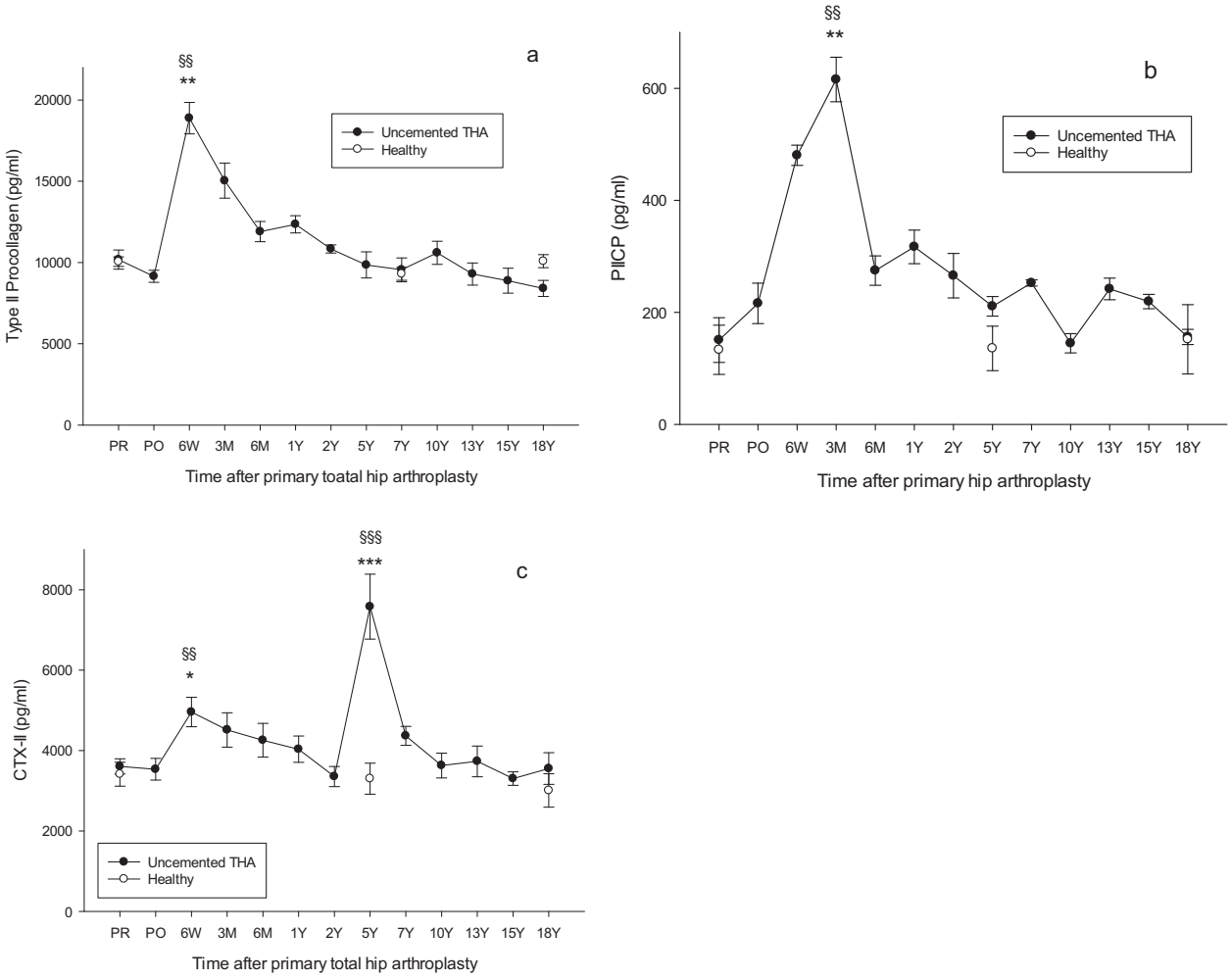

Fig. 1. Markers of cartilage synthesis and turnover in patients with primary total hip arthroplasty (THA) vs healthy (A) Type II procollagen, $^{**}p = 0.004$ THA 6 W vs healthy 58Y, $^{§§}p = 0.002$ THA 6 W vs THA PR (B) PIICP, $^{**}p = 0.003$ THA 3 M vs healthy 58Y, $^{§§}p = 0.005$ THA 3 M vs THA PR (C) CTX-II, $^{*}p = 0.011$ THA 6 W vs healthy 58Y, $^{***}p < 0.001$ THA 5Y vs healthy 67Y, §§ p = 0.002 THA 6 W vs THA PR, $^{§§§}p < 0.001$ THA 5Y vs THA PR. Mean ± SEM.

molecular processes shown in experimental studies of normal fracture healing and by both being short-lived and orderly resolved (Marsell and Einhorn, 2011; Mountziaris and Mikos, 2008). As it is entirely physiological, the secondary proinflammatory and resorptive phase of bone repair would not be expected to present itself with the classical signs of unregulated chronic inflammation (e.g. pain), as would be the case with tissue damaging pathologies, such as excessive micromotion and septic/aseptic loosening (Goodman et al., 2019). The physiologic nature of inflammation during bone repair is further supported by studies showing proinflammatory mediators to be critical for normal osteoblast and chondrocyte differentiation while use of anti-inflammatory drugs has been shown to impair bone healing in both animals and humans (Einhorn, 2002). In addition to being a physiological phase, the transformation of the calcified cartilage callus into bone is a gradual and lengthy process during which the microstructure of the callus is being reorganized with only small changes to total osseous tissue (Gerstenfeld et al., 2006) and thus not likely to be discernable on plain radiographs during osseointegration of hip implants.

4.3. Osteoarthritis

Among the MMPs of the study, only MMP-1 and MMP-9 were higher in presurgery OA relative to healthy, whereas MMP-2 was significantly lower. Although previously considered a non-inflammatory disease related primarily to attrition, new findings show both inflammation and abnormal tissue remodeling to be important mechanisms driving joint degradation in OA, with particular importance assigned to MMP-9 (Troeberg and Nagase, 2012). The latter can however not act alone as the joint cartilage is protected by a layer of aggrecan that needs to be removed by the actions of ADAMTS before enabling collagenases, such as MMP-9, to gain access and degrade collagen fibrils (Troeberg and Nagase, 2012). Such a role was confirmed by augmented levels of ADAMTS4 in presurgery OA patients of the current study. The pathogenic importance of low levels of MMP-2 in presurgery OA patients was highlighted by a hereditary disorder in humans caused by inactivating mutations in the MMP-2 gene (NAO, nodulosis, arthropathy, osteolysis) and reproduced in MMP-2 knockout mice, showing progressive bone loss and joint destruction which are similar to changes caused by overexpressed MMP-9 (Krane and Inada, 2008). Our results showing TIMP-1 to be well above healthy in presurgery OA patients is in line with previous reports linking it to disease progression by showing that high levels of TIMP-1 will slow OA development by acting as a counterweight to excessive MMP-9 (Chevalier et al., 2001). NE and PR3 in pre-THA OA were also significantly above healthy which highlights their role in OA pathology, primarily by being at the frontline of proteolytic activation of the pro-forms of MMP-1, MMP-3 and MMP-9 (Troeberg and Nagase, 2012). A rigorous control of serine protease activity by their endogenous inhibitors is particularly critical as they, in contrast to most MMPs, are active upon secretion (Korkmaz et al., 2008; Korkmaz et al., 2010). Our results showing increased levels of trappin-2/elafin, the potent inhibitor of NE and PR3, in presurgery OA confirm that such a counter-balancing response is present in hip OA patients which is in

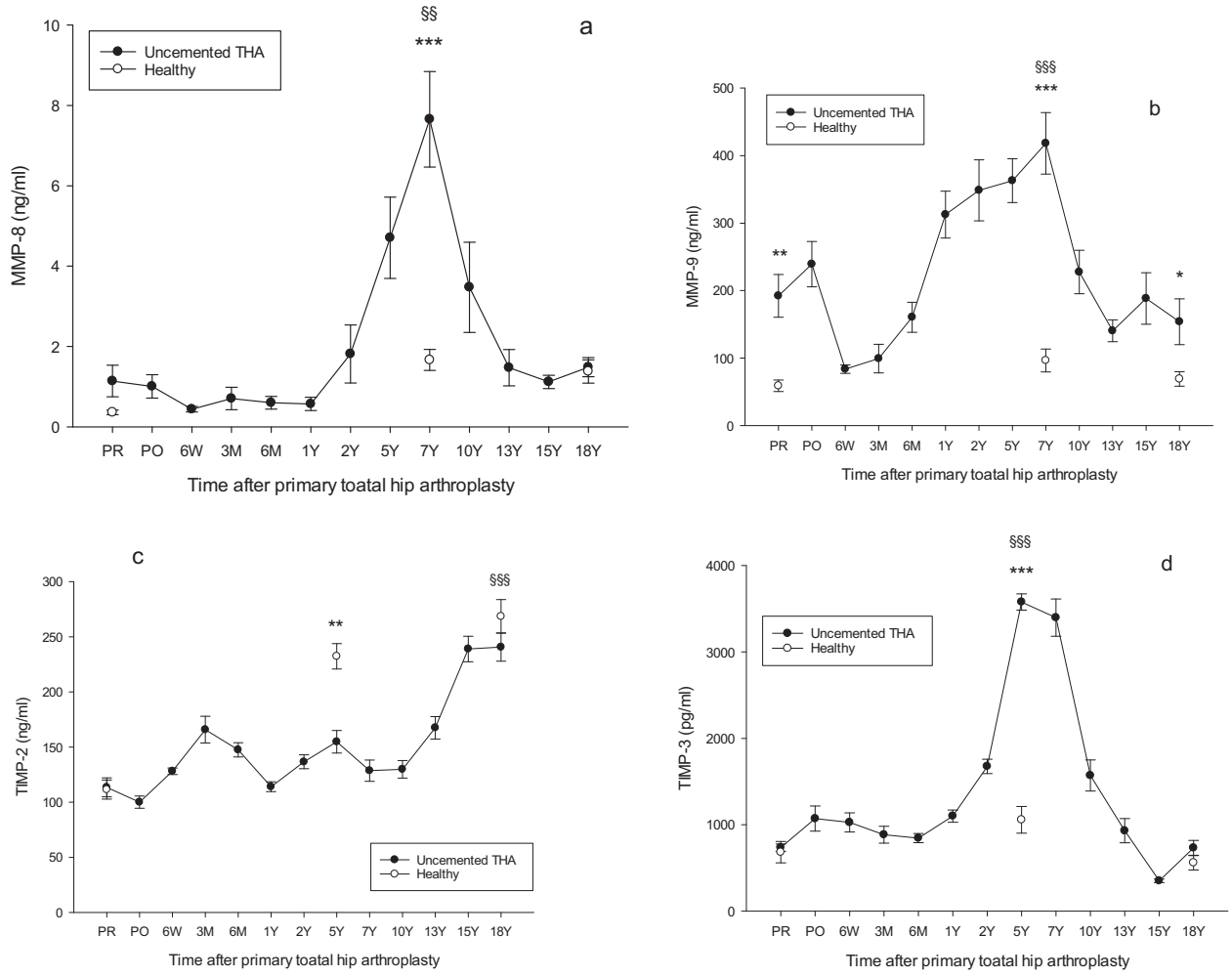

Fig. 2. MMPs and TIMPs in THA patients vs healthy (A) MMP-8, $^{***}p < 0.001$ THA 7Y vs healthy 67Y, $^{\S\S}p = 0.005$ THA 7Y vs THA PR (B) MMP-9, $^{**}p = 0.004$ THA PR vs healthy 58Y, $^{***}p < 0.001$ THA 7Y vs healthy 67Y, $^{*}p = 0.018$ THA 18Y vs healthy 79 years, $^{\S\S\S}p < 0.001$ THA 7Y vs THA PR (C) TIMP-2, $^{**}p = 0.003$ THA 5Y vs healthy 67Y, $^{\S\S\S}p < 0.001$ THA 18Y vs THA PR (D) TIMP-3, $^{***}p < 0.001$ THA 5Y vs healthy 67Y, $^{\S\S\S}p < 0.001$ THA 5Y vs THA PR. Mean ± SEM.

agreement with a previous study showing increased levels of trappin-2 in articular cartilage of knee OA patients (Jaovisidha et al., 2006). Similarly, SLPI, the potent inhibitor of NE, was increased in pre-THA OA being in accordance with an experimental study showing SLPI to increase during the acute and chronic phases of arthritis where it is believed to suppress a critical part of OA pathology, i.e. denaturation of type II collagen (Majchrzak-Gorecka et al., 2016).

Despite having removed the diseased hip joint and repeatedly monitored and excluded patients showing clinical (see HHS) and radiographic signs of OA in the opposite hip joint, the possibility remains that

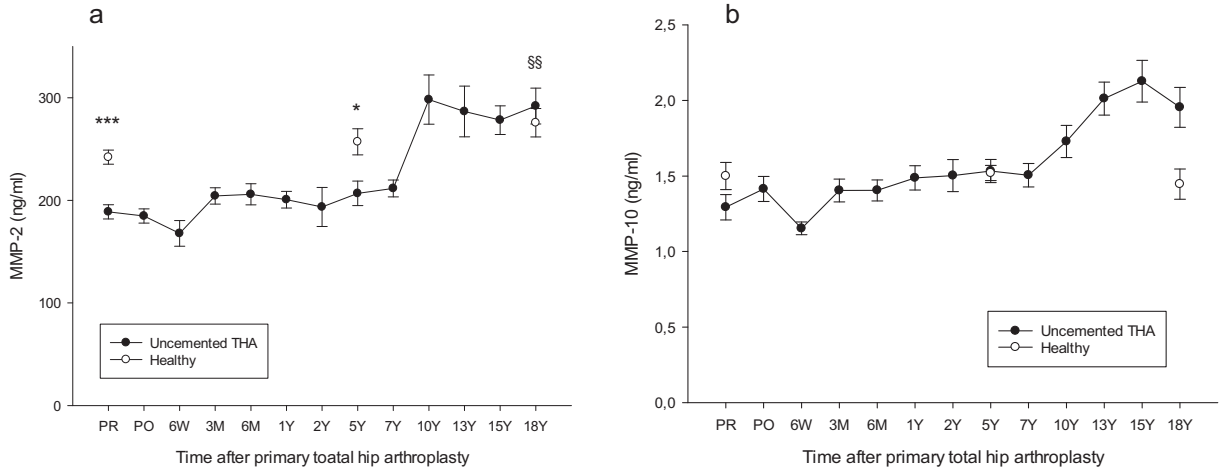

Fig. 3. MMPs in patients with primary total hip arthroplasty vs healthy (A) MMP-2, $^{***}p < 0.001$ THA PR vs healthy 58Y, $^{*}p = 0.011$ THA 5Y vs healthy 67Y, $^{\S\S}p = 0.002$ THA 18Y vs THA PR (B) MMP-10, differences between THA and healthy or within the THA group were not significant. Mean ± SEM.

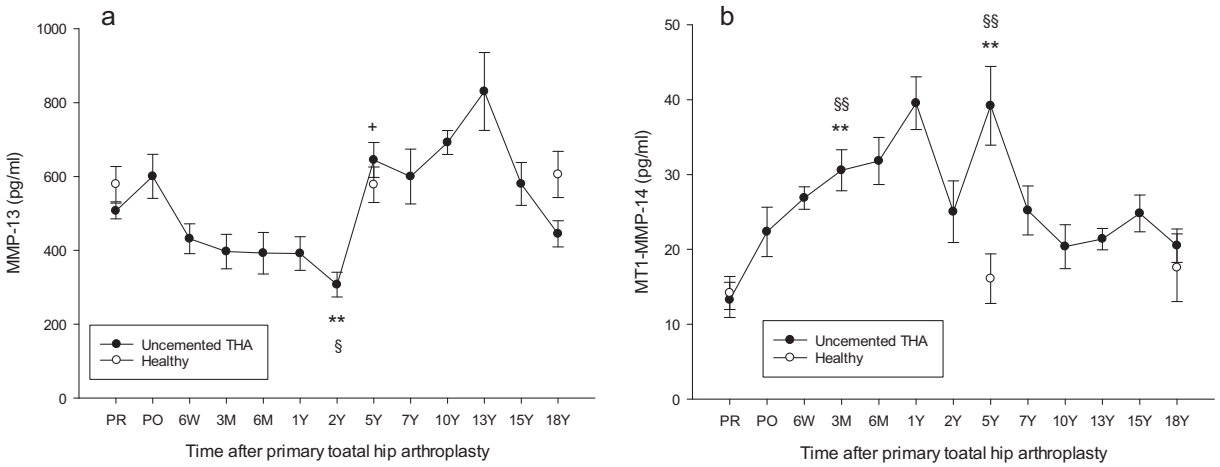

Fig. 4. MMP-13 and MMP-14 in THA patients vs healthy (A) MMP-13, $^{**}p = 0.006$ THA 2Y vs healthy 67Y, $^{\S}p = 0.038$ THA 2Y vs THA PR, $+p = 0.039$ THA 5Y vs THA 2Y (B) MMP-14, $^{**}p = 0.008$ THA 3 M vs healthy 58Y, $^{\S\S}p = 0.006$ THA 3 M vs THA PR, $^{**}p = 0.005$ THA 5Y vs healthy 67Y, $^{\S\S}p = 0.004$ THA 5Y vs THA PR. Mean ± SEM.

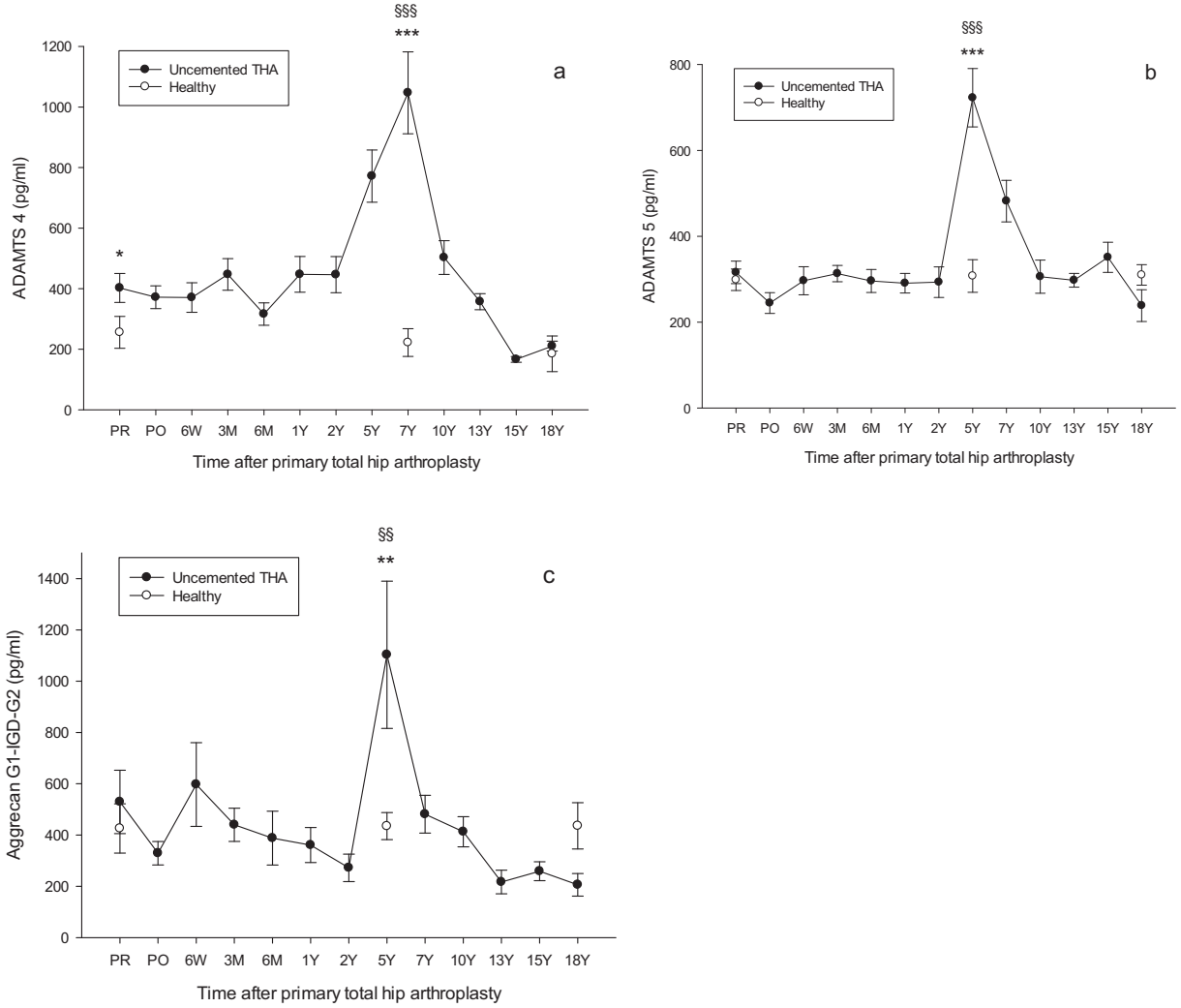

Fig. 5. ADAMTS4, ADAMTS5 and aggrecan fragment (G1-IGD-G2) in THA vs healthy (A) ADAMTS4, $^{*}p = 0.038$ THA PR vs healthy 58Y, $^{***}p < 0.001$ THA 7Y vs healthy 67Y, $^{\S\S\S}p = p < 0.001$ THA 7Y vs THA PR (B) ADAMTS5, $^{***}p < 0.001$ THA 5Y vs healthy 67Y, $^{\S\S\S}p < 0.001$ THA 5Y vs THA PR (C) Aggrecan, $^{**}p = 0.003$ THA 5Y vs healthy 67Y, $^{\S\S}p = 0.009$ THA 5Y vs THA PR. Mean ± SEM.

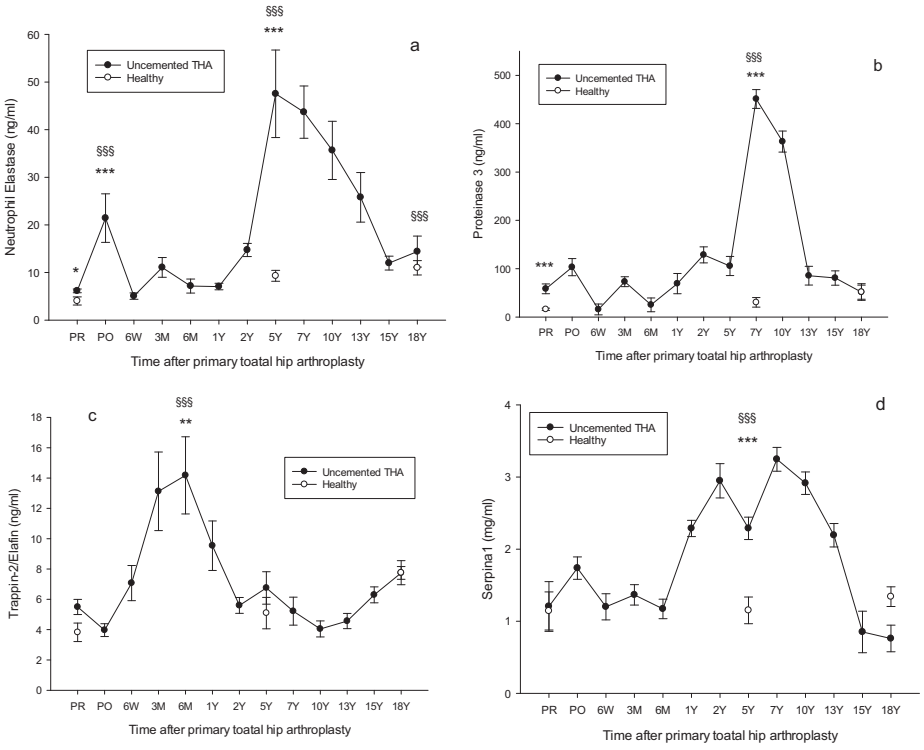

Fig. 6. Serine proteases and their endogenous inhibitors in THA vs healthy (A) Neutrophil elastase, $*p = 0.013$ THA PR vs healthy 58Y, $***p < 0.001$ THA PO vs healthy 58Y and THA 5Y vs healthy 67Y, $^{§§§}p < 0.001$ THA PO vs THA PR, THA 5Y vs THA PR and THA 18Y vs THA PR (B) Proteinase 3, $***p < 0.001$ THA PR vs healthy 58Y and THA 7Y vs healthy 67Y, $^{§§§}p < 0.001$ THA 7Y vs THA PR (C) Trappin-2/elafin, $**p = 0.007$ THA 6 M vs healthy 58Y, $^{§§§}p < 0.001$ THA 6 M vs THA PR (D) Serpina1, $***p < 0.001$ THA 5Y vs healthy 67Y, $^{§§§}p < 0.001$ THA 5Y vs THA PR. Mean ± SEM.

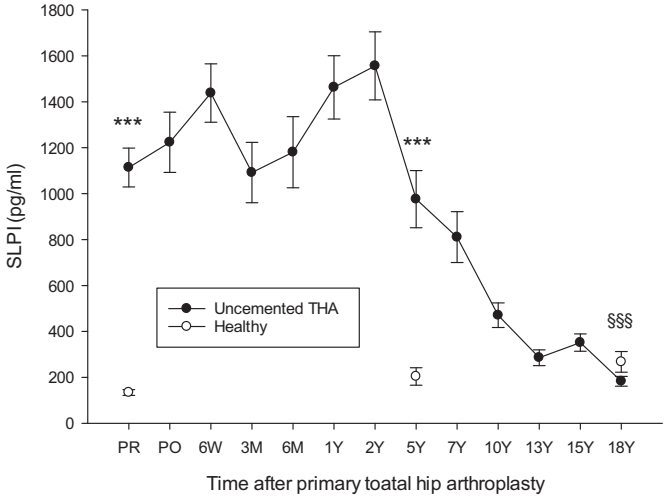

Fig. 7. SLPI in patients with primary total hip arthroplasty vs healthy, $***p < 0.001$ THA PR vs healthy 58Y and THA 5Y vs healthy 67Y, $^{§§§}p < 0.001$ THA 18Y vs THA PR. Mean ± SEM.

patients with asymptomatic OA in other joints could have passed below the radar in the absence of pain, stiffness and aching (Daghestani and Kraus, 2015). As OA is accompanied by a low grade chronic inflammation of the synovial tissues with proinflammatory cytokines being part of the pathogenesis (Troeberg and Nagase, 2012; Daghestani and Kraus, 2015), it could be argued that the peaks of IL-8 and IL-1β seen 5Y post-THA (Cassuto et al., 2017) may be a reflection of asymptomatic OA rather than a natural part of implant osseointegration. The role of IL-1β in OA was made less likely by several studies not supporting it as a tentative disease-related cytokine in hip or knee OA (Sandy et al., 2015) and by studies using joint fluid aspirates and showing concentrations in early and late OA to be essentially identical to levels in normal fluids (Sandy et al., 2015). Moreover, upregulated collagen synthesis in the cartilage of OA patients is not in agreement with the potent anti-chondrogenic effect of IL-1β (Sandy et al., 2015). As IL-8 is almost entirely dependent on IL-1β for its induction (Kaplanski et al., 1994), the increase by IL-8 seen at 5Y post-THA is consequently less likely to be linked to OA. Interestingly, proinflammatory cytokines generally viewed as markers of OA, such as IL-6, TNF-α and CRP (Daghestani and Kraus, 2015), failed to differ between THA and healthy at any point between 6 W and 18Y post-THA (Cassuto et al., 2017). These findings further reduce the likelihood of asymptomatic OA having a bearing on the levels of proinflammatory cytokines and proteases 5Y after THA, even more so when considering the temporary and short-lasting nature of the proinflammatory and protease peaks vis-à-vis the life-long progressive nature of OA (Daghestani and Kraus, 2015).

4.4. Bone healing after hip arthroplasty

Biomolecular processes in the current and previous study (Cassuto et al., 2017) have shown great similarities with the four major phases of endochondral bone repair and will therefore be discussed in that context. MMP-9, by being of particular importance during the entire process of bone healing (Colnot et al., 2003), has been placed at the core of the discussion to serve as a silver thread around which the actions of other mediators are intertwined to create a more comprehensive whole. As the current study emanates from the same cohorts as the previous report (Cassuto et al., 2017) we intend, when important for clarity, to discuss current observations in the context of previous results.

4.4.1. Primary proinflammatory phase

The primary proinflammatory phase triggers the migration and activation of immune cells from the circulation into the area of bone repair where they, aside from countering infections, release proinflammatory cytokines and enzymes capable of digesting and removing damaged tissue (Gerstenfeld et al., 2003). This cleaning activity could explain the paradoxical increase by MMP-1 and MMP-9 on day one post-THA despite that the diseased joint had been removed, a conclusion supported by a study showing high levels of MMP-9 in pre-osteoclasts during the primary proinflammatory phase (Colnot et al., 2003). Similarly, NE and PR3 increased one day after THA, most likely in response to the increased need to degrade ingested host pathogens

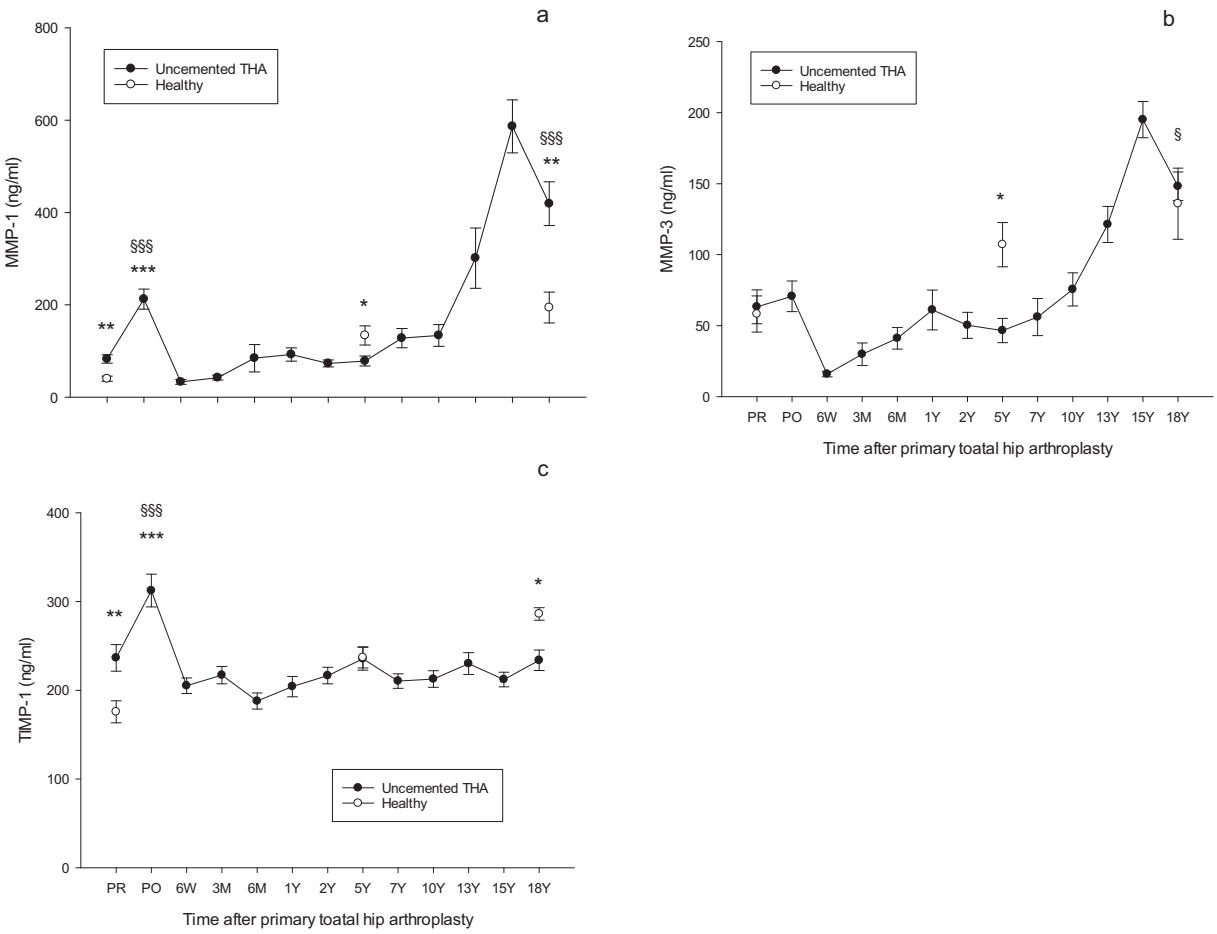

Fig. 8. MMPs and TIMPs in patients with THA vs healthy (A) MMP-1, $^{**}p = 0.009$ THA PR vs healthy 58Y, $^{***}p < 0.001$ THA PO vs healthy 58Y, $^{*}p = 0.018$ THA 5Y vs healthy 67Y, $^{**}p = 0.003$ THA 18Y vs healthy 79Y, §§§p < 0.001 THA PO vs THA PR and THA 18Y vs THA PR (B) MMP-3, $^{*}p = 0.014$ THA 5Y vs healthy 67Y, $^{§}p = 0.026$ THA 18Y vs THA PR (C) TIMP-1, $^{**}p = 0.002$ THA PR vs healthy 58Y, ***p < 0.001 THA PO vs healthy 58Y, $^{*}p = 0.039$ THA 18Y vs healthy 79Y, §§§p < 0.001 THA PO vs THA PR. Mean ± SEM.

and digest damaged ECM after surgery. Increased MMPs in the immediate aftermath to surgical bone trauma could also be linked to the increased synthesis of type I collagen (P1NP) seen immediately after hip arthroplasty (Cassuto et al., 2017) which requires enzymatic processing by MMP-1, able to degrade native type I collagen, and by MMP-9 which digests the solubilized form of type I collagen (Vandooren et al., 2013). The tight regulation of MMPs aimed at safeguarding the integrity of surrounding tissues is consistent with our results showing TIMP-1 to follow on the heels of MMP-1 and MMP-9 on day one post-THA and supported by studies showing TIMP-1 to be secreted in complex with MMP-9 thus allowing for a close regulatory interplay during physiological and pathological ECM remodeling (Vandooren et al., 2013).

4.4.2. Cartilage callus formation and turnover

For type II procollagen to be aggregated and form the collagen fibrils of the osteoid, its amino (N) and carboxyl (C) domains need to be removed (Charni-Ben Tabassi and Garnero, 2007). The resulting by-products, procollagen II C-terminal propeptide (PIICP) and N-terminal propeptide (PIINP), are both produced in a stoichiometric fashion (1:1 ratio to each type II collagen molecule being formed) and released into the circulation where they are extensively used as markers of cartilage synthesis (Charni-Ben Tabassi and Garnero, 2007). Procollagen exists in two subtypes, procollagen IIA, mainly expressed during skeletal development but also found in plasma and used as marker of collagen synthesis, and procollagen IIB, the major form in adult cartilage. The C-terminal propeptide is identical for IIA and IIB (Charni-Ben Tabassi and Garnero, 2007) why we chose PIICP that measures the total of type II collagen synthesis. CTX-II, a byproduct released into the circulation during degradation of type II collagen, was chosen in the current study as a specific marker of cartilage breakdown (Charni-Ben Tabassi and Garnero, 2007). As collagen fibrils of the cartilage callus are arranged in network-like structures, chondrocytes continuously produce large amounts of aggrecan which is embedded between the fibrils and, together with other proteoglycans, forms 90% of the cartilage matrix (Knudson and Knudson, 2001). Our results showing specific markers of type II collagen synthesis, i.e. type II procollagen and PIICP, to increase along with the specific type II collagen degradation marker, CTX-II, confirm that the newly formed cartilagenous callus undergoes turnover as part of the ongoing process of maturation between 6 W and 3 M after surgery. During the continued development of the cartilage callus, proliferative chondrocytes are transformed into the hypertrophic cartilage that gradually becomes mineralized. An interesting observation in this context was made in our previous study (Cassuto et al., 2017) showing osteocalcin (OC) to be two-phased with an initial peak at 6 W post-THA followed by a sharp down-turn at 5 Y and a renewed peak at 13 Y (Fig. 9). OC, first believed to be specific to osteoblasts and a promoter of bone mineralization, was later shown also to be produced by proliferating chondrocytes during mineralization of the hypertrophic cartilage why it is currently used as a marker of both cartilage and bone mineralization (Gerstenfeld and Shapiro, 1996). In agreement with OC, the synthesis of BALP is biphasic (Cassuto et al., 2017) and besides being produced by osteoblasts it is also secreted by hypertrophic chondrocytes (Gerstenfeld and Shapiro, 1996) which would suggest that the two markers act concordantly to induce early cartilage callus

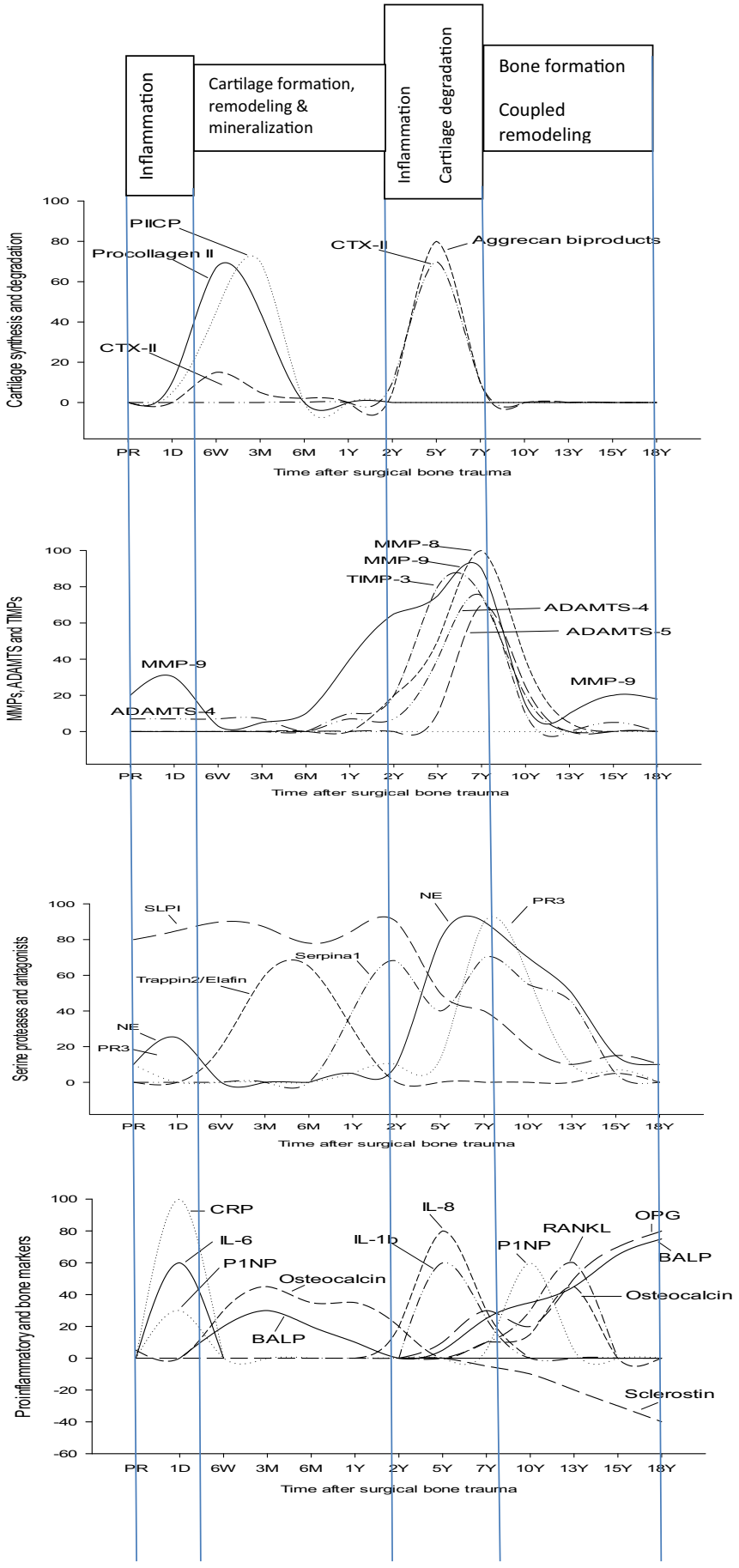

Fig. 9. Schematic summary of the stages of fracture repair and their associated time-induced biomolecular responses, all originating from the current and previous study (the latter represented by the bottom panel and ref. (Cassuto et al., 2017)). High or low plasma levels of individual biomarkers relative to baseline (=PR, preoperative level) are denoted by graphic curves that illustrate their temporal peaks of activity, all being significant relative age-matched healthy control subjects and/or presurgery levels (for details see individual figures). Graphs altitudes are not comparable between individual biomarkers as they are not represented by the actual intensity as percent over baseline. Acronyms: CRP (C-reactive protein, P1NP (N-terminal propeptide of type I collagen), BALP (bone specific alkaline phosphatate), OPG (osteoprotegerin). Other abbreviations are given in the text of the current study.

Table 3
MMPs, ADAMTS, serine proteases and endogenous inhibitors in the three groups of healthy. Mean ± SEM.

Age (Y) mean ± SEM (range)	58 ± 1.7 (40–68)	67 ± 0.6 (61–72)	79 ± 1 (71–88)	*P*-values
PIICP (pg/ml)	133 ± 44	135 ± 40 †	152 ± 63 *§	†*§ = 0.971
Type II Procollagen (pg/ml)	2114 ± 595	2930 ± 401 †	2188 ± 402 *§	†*§ = 0.755
CTX-II (pg/ml)	3412 ± 297	3300 ± 386 †	3008 ± 415 *§	†*§ = 0.897
MMP-1 (ng/ml)	40 ± 6	133 ± 24 †	194 ± 38 *§	† = 0.140 * = 0.185 § = 0.005
MMP-2 (ng/ml)	242 ± 7	257 ± 13 †	275 ± 14 *§	†*§ = 0.076
MMP-3 (ng/ml)	58 ± 12	107 ± 15 †	135 ± 25 *§	† = 0.179 * = 0.323 § = 0.025
MMP-8 (ng/ml)	0.36 ± 0.05	1.6 ± 0.26 †	1.37 ± 0.28 *§	† < 0.001 * = 0.438 § = 0.002
MMP-9 (ng/ml)	90 ± 8	114 ± 25 †	69 ± 12 *§	†*§ = 0.303
MMP-10 (ng/ml)	1.50 ± 0.09	1.52 ± 0.05 †	1.45 ± 0.1 *§	†*§ = 0.772
MMP-13 (pg/ml)	579 ± 47	623 ± 83 †	641 ± 92 *§	†*§ = 0.965
MMP-14 (pg/ml)	14 ± 2	16 ± 3 †	17 ± 5 *§	†*§ = 0.763
TIMP-1 (ng/ml)	175 ± 11	237 ± 11 †	286 ± 16 *§	† = 0.043 * = 0.013 § < 0.001
TIMP-2 (ng/ml)	112 ± 8.9	231.8 ± 29 †	269.6 ± 47 *§	† = 0.011 * = 0.310 § < 0.001
TIMP-3 (pg/ml)	683 ± 124	1056 ± 154 †	560 ± 84 *§	†*§ = 0.260
ADAMTS4 (pg/ml)	256 ± 53	222 ± 46 †	185 ± 59 *§	†*§ = 0.912
ADAMTS5 (pg/ml)	299 ± 25	307 ± 38 †	400 ± 24 *§	†*§ = 0.787
Aggrecan (G1-IGD-G2) (pg/ml)	426 ± 96	435 ± 53 †	436 ± 90 *§	†*§ = 0.886
Neutrophil elastase (ng/ml)	4.0 ± 0.6	9.3 ± 1.4 †	11 ± 1.8 *§	† = 0.004 * = 0.395 § < 0.001
Proteinase 3 (ng/ml)	16 ± 2	30 ± 10 †	52 ± 17 *§	†*§ = 0.562
Serpina1 (mg/ml)	1.14 ± 0.26	1.15 ± 0.18 †	1.34 ± 0.14 *§	†*§ = 0.228
Secretory leukocyte proteinase inhibitor (pg/ml)	134 ± 12	203 ± 38 †	267 ± 45 *§	† = 0.015 * = 0.291 § = 0.191
Trappin-2/Elafin (ng/ml)	3.8 ± 0.8	5.1 ± 1†	7.7 ± 0.8 *§	† = 0.334 * = 0.181 § = 0.018

One-way repeated measures ANOVA for comparison of: † healthy 67 Y versus healthy 58 Y, * healthy 79 Y versus healthy 67 Y and § healthy 79 Y versus healthy 58 Y. †*§ = Inter-group differences not significant.

calcification and late bone mineralization during endochondral healing of hip implants (Fig. 9).

4.4.3. Cartilage callus degradation and remodeling

4.4.3.1. MMP-8 and MMP-9. Our results showing the peak of CTX-II at 5Y to coincide with the peaks of MMP-8 and MMP-9 support this as the phase when the calcified hypertrophic cartilage is degraded and transformed into spongious bone of the hard callus, a conclusion also supported by studies of human articular cartilage showing MMPs, and especially MMP-9, to efficiently cleave type II collagen and release CTX-II (Krane and Inada, 2008; Paiva and Granjeiro, 2014). MMP-9 is important for terminal differentiation of hypertrophic chondrocytes as shown by delayed death of the latter in MMP-9-null mice (Krane and Inada, 2008; Paiva and Granjeiro, 2014) and by studies showing high expression of MMP-9 in chondroclasts and osteoclasts interposed between hypertrophic chondrocytes and newly forming bone (Colnot et al., 2003). Moreover, mice lacking MMP-9 show delayed vascularization of the cartilage, a prerequisite for chondroclast and osteoclast migration and subsequent degradation of the cartilage (Colnot et al., 2003). The mechanisms by which MMP-9 stimulates angiogenesis during the phase of cartilage callus degradation can be multifaceted although proteolytic release of vascular endothelial growth factor (VEGF) from the cartilage matrix is considered the most important as it enables VEGF to attach to receptors on endothelial cells responsible for blood vessel formation (Colnot et al., 2003). Such a relationship was confirmed by results from the current cohort of arthroplasty patients showing a close spatial and temporal relationship between systemic levels of VEGF-A and MMP-9 (unpublished observations). As opposed to a simultaneous increase by MMP-9 and TIMP-1 in presurgery OA and immediately after THA, increased levels of MMP-9 at 5Y post-THA were not paralleled by high TIMP-1. This difference is most likely linked to neutrophils being the prime source of MMP-9 during vascularization of the hypertrophic cartilage as they, opposite to other cells, cannot produce TIMP-1 and thus release TIMP-1-free MMP-9 which is highly pro-angiogenic (Ardi et al., 2007). The concomitant increase of another neutrophil-derived potent inducer of angiogenesis, MMP-8 (Fang et al., 2013), would suggest that the angiogenic activities of both proteases are closely coordinated during the process of cartilage callus remodeling post-THA. As all TIMPs are potent inhibitors of vascular growth (Arpino et al., 2015), they would all be expected to take part in the regulation of MMP-8 and MMP-9. Our results showing suppressed level of TIMP-2 and a highly significant elevation of TIMP-3 would support such role, whereas TIMP-1, which did not differ from healthy, appears only to have a subordinate role during remodeling of the hypertrophic cartilage. A suppression of TIMP-2 could serve to unlock MMP-8 and MMP-9 and allow them to increase sharply whereas TIMP-3, which traces the pro-angiogenic MMPs, is likely to serve as a counter-balancing and fine-tuning regulator of their collagenolytic activity. In addition, TIMP-3 has unique anti-angiogenic capabilities which are not coupled to its anti-MMP activities but rather to its ability to inhibit VEGF signaling by competitively binding to its receptor-2 (Qi et al., 2003) and by promoting vascular endothelial cell apoptosis (Qi and Anand-Apte, 2015). The importance of this anti-angiogenic activity was

demonstrated by a study showing abnormal vascularization in mice lacking TIMP-3 (Janssen et al., 2008). The role of TIMP-3 is however not limited to vascularization as it is also able to exert significant influence on the structure, composition and mechanics of bone tissues by enabling an orderly transition from cartilage to bone (Miller et al., 2017). The latter mechanism is supported by a study in TIMP-3-deficient mice showing poor regulation of collagenases, such as MMP-8 and MMP-9, leading to excessive collagen degradation, exaggerated formation of vascular channels and fragile bone (Miller et al., 2017).

4.4.3.2. MMP-2. In contrast to MMP-9 (gelatinase B), which increased during cartilage callus remodeling, MMP-2 (gelatinase A) was continuously below healthy controls during the entire phase of soft and hard callus formation. The latter finding is in conformity with a study of non-stabilized fractures in mice showing MMP-2 expression to be low throughout the phase of cartilage callus formation and remodeling which led the authors to conclude that it has a subordinate role during endochondral healing (Wigner et al., 2012). The possibility of MMP-2 not simply being by-passed but rather actively suppressed is suggested by a study showing that knockdown of MMP-2 in prechondroblastic mesenchymal cells facilitated their condensation and ensuing chondrogenic differentiation (Malfait et al., 2002). The current study showing TIMP-2 to follow on the heels of MMP-2 by being below healthy throughout the soft and hard callus phases, is also in line with a study in MMP-2 deficient mice showing decreased expression of TIMP-2 (Tortorella et al., 2001). Although MMP-2-null mice with non-stabilized fractures showed no noticeable effect on cartilage development, calcification and remodeling, they did show delayed bone remodeling at later stages (Lieu et al., 2011) which is in line with reports showing MMP-2 deficiency to be associated with reduced proliferation of osteoblasts and osteoclasts (Krane and Inada, 2008; Paiva and Granjeiro, 2014). The above observations could shed light on the sharp increase by MMP-2 and TIMP-2 at 10Y post-THA parallel to a sharp increase by the bone synthesis marker, P1NP (Cassuto et al., 2017).

4.4.3.3. MMP-13. Although MMP-9 can degrade denatured type II collagen, it is unable to cleave native type II collagen (Colnot et al., 2003; Vandooren et al., 2013). This requires activation of another proteinase, MMP-13, able to cleave type II and type X collagen, two major proteins of the cartilage matrix (Behonick et al., 2007). The current study shows MMP-13 in arthroplasty patients to embark on a negative trajectory shortly after surgery and reach a lowest level after 2 Y. This phase of suppressed MMP-13 could be explained by a study in MMP-13-deficient mice showing reduced vascular invasion of the cartilage, reduced chondroclastic presence and consequently delayed apoptosis of hypertrophic chondrocytes (Kosaki et al., 2007). Inhibition of MMP-13 could thus serve as a natural “brake” on cartilage degradation during the period leading to chondrocyte hypermaturity by reducing the lysis of aggrecan and type II collagen. In support, MMP-13-null mice with non-stabilized fractures showed normal formation of hypertrophic cartilage whereas its remodeling and removal was significantly delayed (Behonick et al., 2007). The need to initiate remodeling of the hypertrophic cartilage could thus explain the sharp increase by MMP-13 at 5Y after surgery, albeit to the level of healthy, a conclusion further supported by a previous study showing MMP-13 expression in the hypertrophic cartilage to peak while being resorbed and remodeled (Wigner et al., 2012). The latter study also showed the peak of MMP-13 to be paralleled by high MMP-9 (Wigner et al., 2012) which conforms with the current results and could suggest a regulatory cross-talk between the biomarkers during cartilage callus remodeling post-THA (Vandooren et al., 2013).

4.4.3.4. MMP-14. MMP-14 is a membrane-type collagenase (MT-MMP) which, in contrast to other collagenases that are secreted in soluble form, is anchored to the cell membrane and acts at its surface (Krane and Inada, 2008; Paiva and Granjeiro, 2014). It is often linked to catalytic activation of proMMP-2 although our results showed MMP-2 to be subdued despite high MMP-14 which could be explained by the former also being induced by other MMPs and MT-MMPs (Krane and Inada, 2008; Paiva and Granjeiro, 2014). Interestingly, MMP-14 was the only protease of the study to increase during the early phase of cartilage synthesis and remain elevated throughout the remodeling phase. The importance of MMP-14 during the cartilagenous phase of endochondral bone healing was demonstrated in MT-MMP-deficient mice showing reduced numbers of proliferative chondrocytes, absence of vascular invasion of the hyaline callus, reduced ossification of the cartilage and inadequate remodeling of the ECM (Krane and Inada, 2008; Paiva and Granjeiro, 2014). MMP-14 has also been shown to be an important regulator of MSC commitment during endochondral healing, with low levels inducing chondrocyte formation and high levels directing cells toward the osteoblast line (Krane and Inada, 2008; Paiva and Granjeiro, 2014) which would fit into the current results showing a modest increase by MMP-14 early after hip arthroplasty, when chondrocyte formation is most pronounced, followed by high levels as chondrocytes become hypertrophic, undergo apoptosis and are gradually replaced by osteoblasts.

4.4.3.5. Role of ADAMTS. Studies investigating the formation of aggrecanase-preferred fragments during cartilage degradation have shown ADAMTS4 and ADAMTS5 (ADAMTS4/5) to be responsible for aggrecan degradation in human osteoarthritic cartilage without the participation of MMPs (Malfait et al., 2002). Little is however known of the role played by ADAMTS during endochondral bone repair and their part in the degradation of the hypertrophic cartilage, although ADAMTS4 has been shown to be expressed during murine endochondral cartilage resorption (Wang et al., 2006). The aggrecan molecule is characterized by numerous glycosaminoglycan chains covalently attached to a central core protein having three globular domains (G1, G2 and G3) (Malfait et al., 2002). The peptide region located between G1 and G2 is named interglobular domain or IGD, and contains several cleavage sites for both ADAMTS and MMPs that generate typical neoepitopes following degradation (Malfait et al., 2002). The aggrecan antibody of the current study is directed against recombinant human aa20-675 located in the G1-IGD-G2 domain and, although not directed against a specific epitope, would be expected to recognize both the intact aggrecan molecule and a degradation product containing the aa20-675 segment. Although most MMPs, including MMP-8 and MMP-9, are capable of cleaving aggrecan, albeit at different preferred sites and with lesser potency than ADAMTS (Malfait et al., 2002), our results showing peak levels of ADAMTS4/5, MMP-8 and MMP-9 to coincide with high levels of degradation products of collagen (CTX-II) and aggrecan (G1-IGD-G2) strongly support a concerted action by MMPs and ADAMTS during remodeling of the hypertrophic cartilage. Notably, our results showing TIMP-3 to trace the activities of both ADAMTS4/5 and MMP-8/MMP-9 is in line with its role as a prime regulator of both MMP-8/MMP-9 (Krane and Inada, 2008; Paiva and Granjeiro, 2014) and ADAMTS4/5 (Kelwick et al., 2015), thus extending the coordination between MMPs and ADAMTS during cartilage callus remodeling to also include their mutual inhibitor.

4.4.3.6. Role of serine proteases. Serine proteases are, in similarity with MMPs, tightly regulated by their endogenous inhibitors, the serpins (*SER*ine *P*rotease *IN*hibitors) that, among others, include trappin-2, SLPI and serpina1. After being secreted into the ECM by multiple cell types, trappin-2 is proteolytically processed into two molecules, trappin-2 and elafin, with similar affinity and inhibitory potency for their target molecules (Moreau et al., 2008). As we measured both molecules in the current study, they were collectively named trappin-2/elafin. SLPI, the homologous relative of trappin-2/elafin, blocks serine proteases by attaching to their catalytic center (Moreau et al., 2008), although SLPI is only able to block NE whereas trappin-2/elafin can block both NE and PR3 (Korkmaz et al., 2008; Korkmaz et al., 2010). Serpina1, which is mainly synthesized in the liver and secreted into the

plasma, is a potent inhibitor of both NE and PR3 and acts by luring the proteases to regard it as a substrate and upon attachment trigger their cleavage thereby distorting and rendering them irreversibly inactive (Korkmaz et al., 2008; Korkmaz et al., 2010). As expected, augmented levels of trappin-2/elafin and SLPI were associated with a containment of NE and PR3 between 6 W and 2 Y after THA. Keeping the activities of these potent proteases at bay during the early phase of endochondral healing may serve to safeguard the newly formed cartilagenous tissues from excessive degradation although a highly significant elevation of SLPI throughout the soft callus phase could, besides being anti-protease, also prevent premature accumulation of leukocytes in the repair tissues previously shown in SLPI-deficient mice to significantly impair healing (Majchrzak-Gorecka et al., 2016; Ashcroft et al., 2000). This leukocyte suppressive effect of SLPI could explain its sharp drop 5Y post-THA when there is a need for increased influx of neutrophils to the bone repair site during the start of the secondary proinflammatory phase of implant osseointegration, as signaled by a sharp increase in the level of the potent neutrophil chemoattractant IL-8 (Cassuto et al., 2017) and in accordance with a study showing increased production of IL-8 in SLPI-deficient mice (Majchrzak-Gorecka et al., 2016).

While SLPI decreased at 5Y, another serine protease inhibitor, serpina1, increased sharply on the heels of its target enzymes, NE and PR3, thus supporting it as instrumental in the regulation and fine-tuning of serine protease activities during the phase of cartilage resorption and primary bone formation. The remarkable alignment of NE/PR3 with augmented levels of MMP-8/MMP-9 at 5–7 Y post-THA clearly suggests that they act in concert to degrade and remodel the cartilage callus although potential redundancies may exist as they are able to process common substrates of the ECM (Krane and Inada, 2008; Paiva and Granjeiro, 2014; Korkmaz et al., 2008; Korkmaz et al., 2010). Such cross-talk is supported by a study showing that NE can activate MMP-9 and at the same time inactivate its inhibitor, TIMP-1, while both MMP-8 and MMP-9 are able to inactivate serpina1, the inhibitor of NE and PR3 (Korkmaz et al., 2010), thereby mutually amplifying their cycle of proteolytic activity during angiogenesis and cell migration.

4.4.4. Secondary proinflammatory phase

4.4.4.1. Role of MMPs.

The secondary proinflammatory phase 5-7Y after hip arthroplasty showed high levels of IL-8 and IL-1β (Cassuto et al., 2017). The former is mandatory for the recruitment of neutrophils to the site of reparative bone tissue where they are activated and release their granules containing proteases, primarily MMP-8, MMP-9 and serine proteinases, which allows them to digest their way to the repair site (Van Den Steen et al., 2003). MMP-9 is particularly important for neutrophil migration as it, besides digesting the basal membrane and ECM, also increases capillary permeability at the site of inflammation (Vandooren et al., 2013). Our results showing a striking similarity, temporal and spatial, between the 5Y levels of MMP-8/MMP-9, on one hand, and IL-8/IL-1β (Cassuto et al., 2017), on the other, would support a coordinated action during the secondary proinflammatory/resorptive phase. Such close interaction is illustrated by a study showing that a positive feedback loop exists between IL-8 and MMP-9, allowing the protease to cleave IL-8 and increase its potency up to 30-fold while IL-8, in turn, can stimulate the release of MMP-9 from neutrophils thereby jointly boosting the recruitment of neutrophils and their enzymatic activities (Van Den Steen et al., 2003). The importance of this interplay was further highlighted by a study showing that anti-MMP-9 antibodies were able to completely prevent IL-8-induced mobilization of pre-neutrophil hematopoietic stem cells from the bone marrow (Pruijt et al., 1999). In similarity with IL-8, IL-1β is a potent inducer of MMP-9 in neutrophils and thus an initiator of MMP-9-induced angiogenesis and degradation of the hypertrophic cartilage (Vu et al., 1998). By also being able to direct the differentiation of MSCs toward osteoblasts in replacement of apoptotic chondrocytes, IL-1β has another critical role during the transformation of cartilage callus to bone (Mumme et al., 2012).

4.4.4.2. Role of ADAMTS.

The coinciding peaks of ADAMTS4/5 and IL-1β (Cassuto et al., 2017) 5 Y post-THA are in line with a study showing IL-1β to activate ADAMTS4/5 in chondrocytes (Tortorella et al., 2001) lending further support to a close coordination between the proteases and proinflammatory cytokines during endochondral bone repair.

4.4.4.3. Role of serine proteases.

In similarity with MMP-8/MMP-9 and ADAMTS4/5, the peaks of NE and PR3 coincided with augmented levels of IL-8 and IL-1β (Cassuto et al., 2017) suggesting that a similar cross-talk exists between serine proteases and proinflammatory cytokines. In support, neutrophils exposed to IL-8 have been shown to release NE and PR3 which, in turn, induce release and activation of IL-8 thereby triggering a perpetual cycle of neutrophil recruitment and activation (Korkmaz et al., 2008; Korkmaz et al., 2010). The inflammatory cascade is also propelled by serine protease-induced transformation of progranulin, a potent anti-inflammatory growth factor, into the pro-inflammatory granulin, which is a strong inducer of IL-8 release from granulocytes (Korkmaz et al., 2008; Korkmaz et al., 2010). A study showing serpina1 to inhibit IL-8-induced mobilization of leukocyte precursors from the bone marrow (van Pel et al., 2006) lend further support to the importance of such cross-talk for the regulation of white cell mobilization during the second proinflammatory and resorptive phase of osseointegration.

4.4.5. Formation of bone and coupled bone remodeling

The current results showing MMP-9 to remain above healthy until 18 Y and a previous experimental study showing elevated MMP-9 to persist throughout the course of endochondral bone repair (Colnot et al., 2003), support the importance of MMP-9 beyond the cartilage remodeling phase of implant osseointegration. The temporary structures driving bone remodeling are known as basic multicellular units (BMUs) as they comprise of osteoclasts, osteoblasts, osteocytes, bone lining cells and reversal cells (Raggatt and Partridge, 2010). To enable migration of cells into the BMUs and provide them with nutrients and signaling molecules, ingrowth of capillaries is required (Raggatt and Partridge, 2010). This process is likely to be driven by MMP-9 as tissues lacking MMP-9 show failure of pre-osteoclasts to transmigrate from the bone marrow and circulation into the area of bone repair (Colnot et al., 2003). Moreover, once the differentiation of pre-osteoclasts into mature osteoclasts has been initiated by RANKL, these osteoclasts continuously produce MMP-9 which is indispensable for their continued migration through the ECM (Vandooren et al., 2013). The cellular responses of an active BMU are divided into a sequence of events named *activation, resorption, reversal, formation and termination* phases (Raggatt and Partridge, 2010). During the *activation* phase, osteoblasts secrete RANKL which attaches to preosteoclasts and trigger their differentiation into mature osteoclasts, a phase reaching a peak 13 Y after hip arthroplasty (Cassuto et al., 2017). Osteoclasts are however unable to undertake resorptive activity as long as unmineralized osteoid covers the surface of the BMUs (Raggatt and Partridge, 2010). By releasing collagenases, such as MMP-9, osteoblasts and stromal fibroblasts are able to digest the osteoid and expose the underlying mineralized bone thus enabling osteoclasts to attach and initiate the subsequent phase of *resorption* (Raggatt and Partridge, 2010). Despite having terminated their activity and moved on, osteoclasts leave behind collagen matrix debris which triggers the ensuing *reversal* phase during which undefined mononuclear cells (possibly macrophages) remove remnants of collagen from the surface and prepare it for subsequent osteoblast-mediated bone formation (Raggatt and Partridge, 2010). The latter process requires that reversal cells release collagenolytic proteases, such as MMP-1, able to degrade native type I collagen, and MMP-9, able to digest the solubilized form of type I collagen (Vandooren et al., 2013). The current results showing augmented levels of MMP-9 and MMP-1 13-18Y after THA paralleled by suppressed level of their common inhibitor, TIMP-1, is thus in line with their important role during bone remodeling.

4.5. Understanding prosthesis loosening in the context of the current results

It is our belief that the most prevalent view among orthopedic surgeons, based primarily on radiological findings and perceived early stiffness of periimplant tissues, is that hip implants reach a final stage of bone healing within two years of the index operation. This has left us with a narrow path when it comes to explaining why implants fail following this 2-year phase, as the only viable alternative would be limited to mechanisms that actively dissolve the "mature" bone surrounding the implant, such as infection, wear particles, stress-shielding and micromotion (Sundfeldt et al., 2006). However, the current and previous study (Cassuto et al., 2017) showing a decade-long process before successfully integrated hip implants reach a phase of coupled bone remodeling opens up for additional, although less investigated, alternatives to implant failure such as age, nutrition, diseases and medications, all having the potential of negatively affecting the skeleton and derailing the normal process of bone healing. Accumulation of wear particles in the vicinity of implants has been at the forefront of the mechanisms believed to cause periimplant osteolysis. However, extensive research efforts have provided a valuable window into the complex responses of immune and bone regenerative cells to wear particles that may pose significant challenges to implant survival, not only by triggering bone resorption, but also by interfering with the differentiation, maturation and function of osteoprogenitor cells critical to both initial osseointegration of implants and the continuous remodeling of bone in the implant perimeter throughout its lifetime (Goodman et al., 2006). Although implant designs and biocompatibility have undergone significant improvements in recent years, the question of how biology responds to insertion of a shaft into one of the most immune-active sites of the body, the femoral bone marrow, is still unclear. This was highlighted in a recent review on the implications of stress-shielding for the long-term fixation of hip implants where the author stresses that although stress-shielding can be influenced by implant design, the biological response of the host tissue to the presence of the implant is critical to its long-term success (Sumner, 2015). The ability of the periprosthetic regenerative tissues to overcome challenges, albeit at a low pace, is illustrated by the undisputed fact that a vast majority of hip implants osseointegrate successfully despite a lifelong accumulation of wear in the vicinity of all implants. Understanding how implants are integrated is therefore critical for our ability to decipher important mechanisms causing implants to fail. The ability of many biomarkers, such as proinflammatory cytokines, RANKL, MMPs, ADAMTS and serine proteases of the previous (Cassuto et al., 2017) and current study to be of critical importance for normal bone healing (Marsell and Einhorn, 2011; Mountziaris and Mikos, 2008; Gerstenfeld et al., 2003; Cox et al., 2010) while at the same time have the ability to induce osteolysis (Krane and Inada, 2008; Paiva and Granjeiro, 2014; Korkmaz et al., 2008; Korkmaz et al., 2010) and be implicated in prosthesis loosening (Syggelos et al., 2013), illustrates the delicate balance between the good and the bad during osseointegration of implants.

4.6. Limitations

There are several limitations to the current study. First, only limited demographic, clinical and drug use data are reported in the THA and healthy cohorts. Drug use data in THA patients was only reported for drugs known to affect bone metabolism, such as steroids, immunotherapy or bone-regulating drugs (e.g. bisphosphonates, denosumab, calcitonin, PTH). Three patients on prohibited drugs (bisphosphonates) were identified, albeit excluded on other criteria. Aside from a history of recent bone trauma, no explicit medical and drug use data other than those being used for exclusion of THA patients were considered when recruiting healthy subjects. Second, we measured biomarkers in plasma that do not only reflect changes related to the implant as they may also originate from other parts of the musculoskeletal system. We believe however that the latter changes would be equally reflected in the population of gender/age-matched healthy controls. Third, recruitment of healthy controls and OA patients awaiting THA was done 10-13Y after inclusion of arthroplasty patients was terminated. The strength of the study is that biomarkers were sampled during two decades in a single population of hip arthroplasty patients and analyzed on a high-sensitivity and wide-dynamic range platform allowing for detection of minor changes with high accuracy. Future longitudinal studies with a follow-up of at least 5–7 years, thus enabling them to capture the bulk of endochondral callus remodeling, will be needed to verify the reproducibility of the current results and serve as proof of concept. Such studies should rely on biomarkers from different cohorts of hip arthroplasty patients and gender/age matched healthy controls and would benefit from inclusion of additional metrics of bone remodeling.

Disclosures

The authors have nothing to disclose.

CRediT authorship contribution statement

JC contributed to research design, acquisition of blood samples, analysis of biomarkers, analysis and interpretation of data and drafting of the paper. AF contributed to research design and critically revised the manuscript. JG contributed to research design, analyzed radiological data and critically revised the manuscript. HM and JK contributed to research design, acquisition of blood samples and critical revision of the manuscript. All authors have read and approved the final version of the manuscript.

Declaration of competing interest

The authors have no conflict of interest to report.

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