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Effects of coagulation factors on bone cells and consequences of their absence in haemophilia a patients

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Haemophilia is associated with reduced bone mass and mineral density. Due to the rarity of the disease and the heterogeneity among the studies, the pathogenesis of bone loss is still under investigation. We studied the effects of coagulation factors on bone cells and characterized in a pilot study the osteoclastogenic potential of patients' osteoclast precursors. To evaluate the effect of coagulation factors on osteoclasts, we treated Healthy Donor-Peripheral Blood Mononuclear Cells (HD-PBMC) with Factor VIII (FVIII), von Willebrand Factor (VWF), FVIII/VWF complex, activated Factor IX (FIXa), activated Factor X (FXa) and Thrombin (THB). FVIII, VWF, FVIII/VWF, FXa and THB treatments reduced osteoclast differentiation of HD-PBMC and VWF affected also bone resorption. Interestingly, PBMC isolated from patients with moderate/severe haemophilia showed an increased osteoclastogenic potential due to the alteration of osteoclast precursors. Moreover, increased expression of genes involved in osteoclast differentiation/activity was revealed in osteoclasts of an adult patient with moderate haemophilia. Control osteoblasts treated with the coagulation factors showed that FVIII and VWF reduced ALP positivity; the opposite effect was observed following THB treatment. Moreover, FVIII, VWF and FVIII/VWF reduced mineralization ability. These results could be important to understand how coagulation factors deficiency influences bone remodeling activity in haemophilia.

Keywords Bone diseases, Coagulation factors, Haemophilia A, Inherited coagulation disorders, Rare disease

To date, clinicians are managing patients with haemophilia (PWH) with several comorbidities, of which chronic arthropathy are the most prevalent[1–5]. It has been reported that ~85% of hemorrhages occur in the musculoskeletal system, impacting the already unstable skeletal condition[6]. Increased fracture risk and decreased bone mass and bone mineral density (BMD) have also been reported in PWH[7–9]. Even if the different prevalence of low BMD in patients affected by severe, moderate, and mild forms of the disease has not been reported yet[10,11], it has been already assessed that the percentage of males with reduced bone mass among subjects with haemophilia A is more than in the normal population[12]. Likewise, animal studies showed the occurrence of reduced trabecular bone mass in a mouse model of FVIII deficiency[13]. The pathogenesis of low BMD associated

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with haemophilia is not completely understood, although increasing numbers of studies are emerging with variable findings[13–22]. Interestingly, it was demonstrated that BMD in patients with severe haemophilia and subjected to prophylaxis with replacement factor concentrates was similar to that revealed in patients with mild haemophilia without prophylaxis[23]. One of the most predisposing factors associated with bone alterations in haemophilia A has been proposed to stem from inactivity and lack of weight-bearing exercise associated also with low levels of vitamin D, increased urinary calcium loss and virus infection[10,15,17,24–28]. Moreover, in PWH low BMD was associated with reduced levels of both osteocalcin and bone resorption marker carboxy-terminal collagen crosslinks (CTX), suggesting reduced bone remodeling activity[29]. In other studies, contrasting results were obtained on the circulating and synovium-associated levels of both osteoclastogenic cytokine Receptor Activator of NF-κB Ligand (RANK-L) and its receptor decoy osteoprotegerin (OPG) in PWH with respect to BMD T-score of lumbar spine[30,31]. Pharmacological treatments of reduced BMD and fractures in PWH include antiresorptive and anabolic drugs[32]. However, Strauss et al. reported that 16 patients receiving anti-osteoporotic therapy did not show any significant changes of BMD in a follow-up period of about 13 years[17].

From these data, it emerges that an unequivocal risk factor has not been identified as mostly responsible for the reduced BMD observed in PWH. Hence, we have decided to investigate the pathogenesis of this phenomenon by evaluating the effect of various coagulation factors on the main actors of bone physiology, that are bone-resorbing osteoclasts and bone-forming osteoblasts. Alterations of the osteoclast pathway regulated by the main osteoclastogenic cytokine RANK-L and its decoy receptor OPG have been suggested to be involved in haemophilia[16]. A direct interaction of the FVIII/VWF complex with RANK-L and OPG has been demonstrated[21]. Indeed, surface plasmon resonance experiments revealed that the FVIII/VWF complex binds RANK-L and OPG through VWF, establishing an interaction between the three molecules[21]. Thus, the FVIII/VWF complex increases the affinity between RANK-L and OPG, facilitating its inhibitory activity[15,16]. Moreover, previous studies showed a positive, non-enzymatic effect of thrombin on osteoclast differentiation[33].

In this study, we extensively investigated the in vitro direct effect of several coagulation factors [FVIII, VWF, FVIII/VWF, FIXa, FXa and thrombin (THB)] on osteoclasts and osteoblasts and the repercussions of non-replacing therapies (emicizumab and denecimig antibodies) on monocyte subsets precursors of osteoclasts, exploring possible cellular mechanisms responsible for the perturbation of bone remodeling activity observed in PWH.

Results

In vitro effects of coagulation factors on osteoclasts

To determine whether the reduced bone mass observed in patients could depend also on the primary effect of coagulation factors' deficiency on bone cells, we studied their effects on osteoclasts and osteoblasts. Osteoclastogenesis assays were performed using HD-PBMC induced to differentiate into mature osteoclasts in the presence of recombinant M-CSF (Macrophage Colony-Stimulating Factor), RANK-L and, according to previous studies[34,35], with saturating concentrations of FVIII, VWF, and FVIII/VWF complex, with or without OPG. As expected, OPG alone inhibited osteoclast differentiation; the treatment with FVIII, VWF and FVIII/VWF complex reduced osteoclastogenesis by ~ 20%, 43% and 50% levels, respectively (Fig. 1a,b). The combined treatment with OPG showed a trend of exacerbated inhibition although not significant compared to the coagulation factor alone (Fig. 1a,b). However, no differences in number of nuclei *per* osteoclast were observed (Fig. 1c). To investigate the effect of late mediators of the coagulation cascade on osteoclast differentiation, HD-PBMC were also treated with FIXa, FXa and THB. We perfomed concentration-dependent experiments with these coagulation factors. While no alterations of osteoclastogenesis were observed following treatment with FIXa (see Supplementary Fig. S1a,b online), FXa reduced about 50% of the osteoclast differentiation at 10 nM (Fig. 1d,e), and no further decrease was observed at higher concentrations (see Supplementary Fig. S1c,d online). Moreover, about 70% reduction of osteoclast differentiation was revealed following the treatment with 50 nM thrombin (Fig. 1d,e); a trend of further decrease was observed in the presence of 100 nM THB (see Supplementary Fig. S1e,f online). Regarding the effects on osteoclast activity, VWF treatment with a concentration of 20 µg/ml (≅ 200 U/dl) significantly reduced about of 75% the bone resorption activity (Bone resorption vs. control (%): PBS: 102.30 ± 5.18; VWF: 25.85 ± 24.25. $p = 0.0008$); no significant effect was observed with FVIII, FVIII/VWF, FIXa, FXa and THB treatment (see Supplementary Fig. S1g online).

Increased osteoclastogenesis in patients with moderate and severe haemophilia

Based on the results obtained with coagulation factors-treated HD-PBMC, we aimed to validate them also in a pilot *proof-of-concept* study using ex vivo samples from PWH with different ages, treatment and disease severity. Thus, we evaluated the osteoclastogenic potential of PBMC isolated from patients with haemophilia A to understand whether Factor VIII deficiency could directly affect osteoclast formation. Patient- and HD-PBMC were differentiated into TRAcP (Tartrate Resistant Acid Phosphatase) positive multinucleated cells in the presence of M-CSF and RANK-L. In accordance with results shown in Fig. 1, we observed more mature TRAcP positive osteoclasts in cultures obtained from off-therapy adult patient with moderate haemophilia (FVIII = 4.4 U/dl) (Patient #1, Table 1) when compared to age-matched controls (Fig. 2a,b). Gene expression analysis performed on RNA extracted from osteoclasts of the patient showed increased mRNA levels of *RANK*, *TRAF6* (TNF receptor-associated factor 6), *CTSK* (Cathepsin K) and *TCIRG1* (T Cell Immune Regulator 1) genes, which encode proteins involved in osteoclast differentiation and activity (Fig. 2c). Moreover, also PBMC isolated from an untreated pediatric patient with severe haemophilia A (Patient #2, Table 1), showed an increase of osteoclast formation ability (Fig. 2d,e). Interestingly, PBMC isolated from the same patient after 6 months of treatment with emicizumab, a monoclonal antibody mimicking the function of FVIII in the activation of FX by FIXa[36], revealed ~ 90% reduction of the ability to differentiate into mature osteoclasts (Fig. 2d,e). On the other

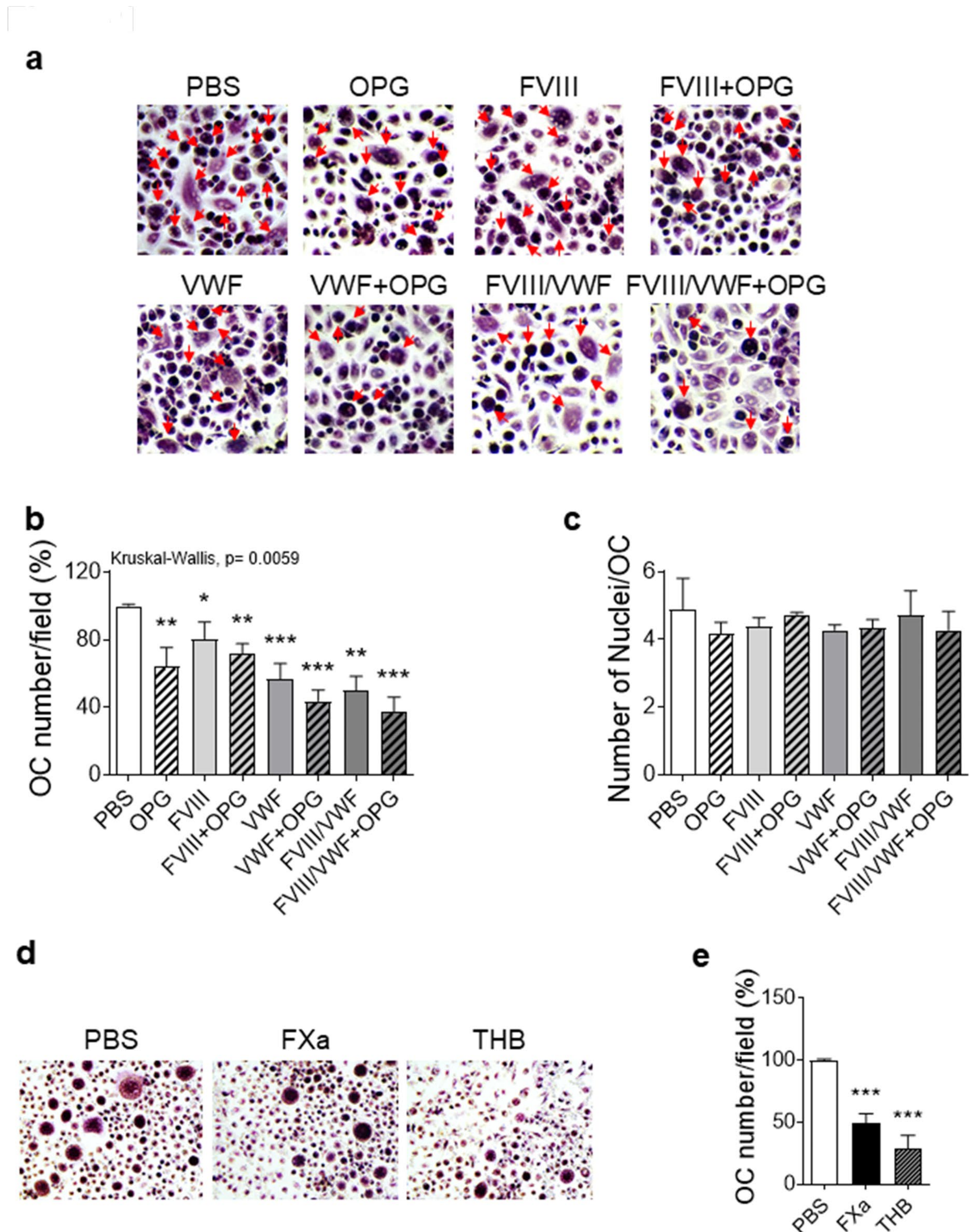

Fig. 1. In vitro effects of coagulation factors on osteoclastogenesis. (**a**–**c**) Peripheral Blood Mononuclear Cells isolated from 3 Healthy Donors (HD-PBMC) were cultured for 14 days in presence of M-CSF (20 ng/ml)/RANK-L (30 ng/ml) and PBS or OPG (25 ng/ml), FVIII (2 U/ml), VWF (20 µg/ml ≅ 200 U/dl), FVIII/VWF complex (2 U/ml). (**a**) Representative pictures of osteoclast cultures stained for TRAcP. Original magnification: 10×. Red arrows indicate multinucleated (≥ 3 nuclei) TRAcP positive osteoclasts. (**b**) Number of osteoclasts (OC) *per* field and (**c**) number of nuclei *per* osteoclast. (**d**) TRAcP staining of HD-PBMC cultured for 14 days in presence of M-CSF (20 ng/ml)/RANK-L (30 ng/ml) and PBS or activated factor X (FXa, 10 nM) and thrombin (THB, 50 nM). Original magnification: 10×. (**e**) Number of osteoclasts (OC) *per* field. Results are expressed as percentage of osteoclasts vs. PBS-treated cells and as mean ± SD of at least three independent experiments. p-values are reported as each treatment compared to PBS. $^{*}p \le 0.05$, $^{**}p \le 0.01$ and $^{***}p \le 0.001$.

Patient	Age	FVIII (U/dl)	HJHS ScoreJ HS score	History of inhibitors	Viral infections	Therapy	Joint bleeds
#1	43 years	4.4	4	Negative	Negative	On demand (Moroctocog alfa)	Knees hemarthr. (4 episodes)
#2	15 months	<1	N.D.	Negative	Negative	Emicizumab	0
#3	14 years	8	1	Negative	Negative	On demand (Octocog alfa)	2 episodes of bleeding
#4	14 years	28	0	Negative	Negative	On demand (Octocog alfa)	0
#5	19 years	40[§]	0	Negative	Negative	On demand (Octocog alfa)	0
#6	6 years	<1	N.D.	Negative	Negative	Denecimig	0

Table 1. Clinical description of PWH.
FVIII coagulation Factor VIII, *HJHS* health joint haemophilia score, *N.D.* not determined. [§]The mild haemophilia form was confirmed by the identification of the *FVIII* gene mutation c.460, A>G, responsible for the aminoacid substitution p.Thr135Ala[64]. The VWF: Ag level was 78 U/dl.

hand, cells isolated from adolescent patients affected by mild forms of the disease (FVIII levels: 8 and 40 U/dl, respectively) (patients #3, #4 and #5, Table 1) did not show alterations of osteoclastogenesis (Fig. 2f).

Evaluation of osteoclast precursor populations in patients

To dissect the mechanisms related to the increased osteoclastogenesis observed in PBMC isolated from moderate and severe PWH, we analyzed the circulating population of osteoclast precursors ($CD14^{+}CD11b^{+}/CD45^{+}$ cells) in patients' PBMC. Cytofluorimetric analysis (see Supplementary Fig. S2 online for FACS gating strategy) highlighted no quantitative differences of $CD14^{+}CD11b^{+}/CD45^{+}$ cell population between HD and adult moderate PWH (patient #1, see Table 1) (Fig. 3a,b); on the other hand, a trend of increase was observed in the untreated pediatric severe PWH (patient #2, Table 1), whereas following emicizumab treatment $CD14^{+}CD11b^{+}/CD45^{+}$ cell population was reduced (Fig. 3a,c, triangle). Interestingly, the other pediatric patient #6 treated with denecimig, a novel bispecific antibody that assembles with activated coagulation FIXa and FX, showed levels similar to those observed in healthy donors (Fig. 3c, circled triangle). Moreover, we evaluated the expression of osteoclast precursor markers including CD115 (also known as M-CSF receptor, M-CSFR). Nevertheless, the adult moderate PWH (patient #1, see Table 1), being off-therapy, exhibited an increase in $CD14^{+}CD115^{+}/CD45^{+}$ cell population when compared to controls (Fig. 3d,e). Interestingly, the pediatric patient with severe haemophilia before any prophylactic therapy mirrored this trend, and after treatment with emicizumab the cell population number was restored to control levels (Fig. 3d,f). The patient #6 treated with denecimig showed levels of $CD14^{+}CD115^{+}/CD45^{+}$ cells similar to those observed in healthy donors (Fig. 3f, circled triangle). No differences of $CD14^{+}CD11b^{+}/CD45^{+}$ and $CD14^{+}CD115^{+}/CD45^{+}$ cell populations were observed in adolescent patients with mild haemophilia (see Supplementary Fig. S3a–d online).

Monocyte subsets distribution in patients

To study a possible correlation between monocytes and osteoclast formation in PWH, we performed FACS analysis of monocyte subsets in HD and patients, analyzing also $CD16^{-}$ cells characterized by an increased ability to differentiate into osteoclasts[37]. As shown in Fig. 4, we characterized three different sub-populations inside the monocyte gate: classical ($CD16^{-}CD14^{++}$), intermediate ($CD16^{+}CD14^{++}$), and non-classical ($CD16^{++}CD14^{+}$)[38].

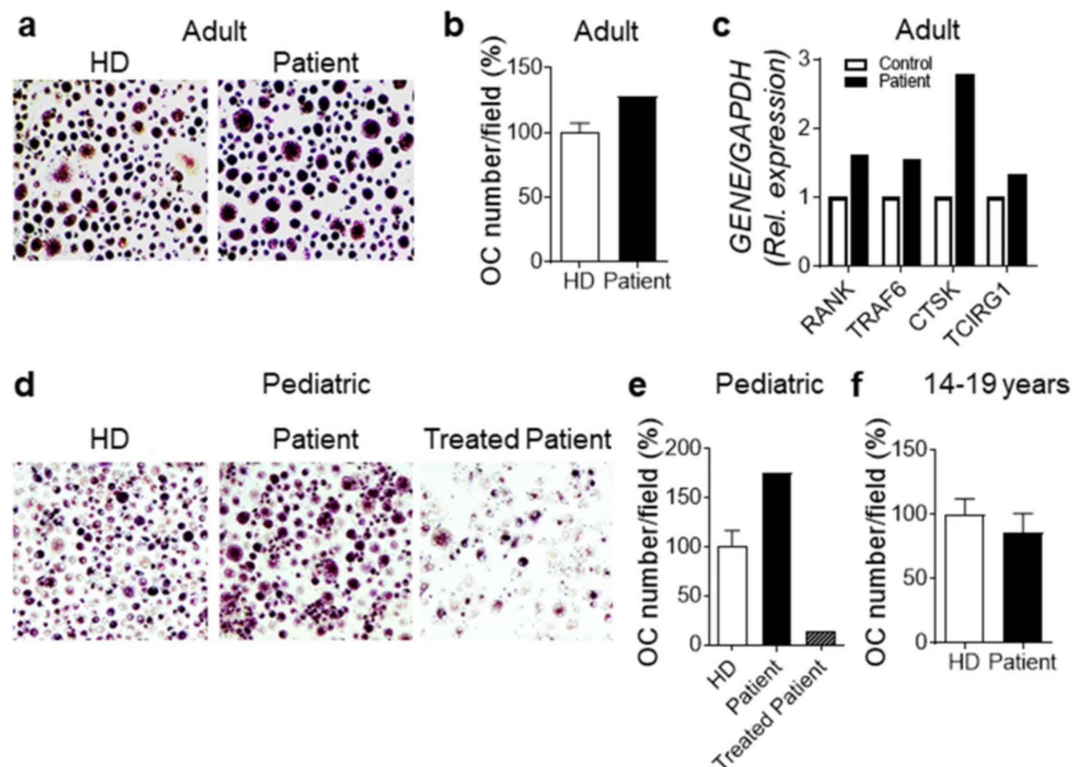

Fig. 2. Increased osteoclastogenesis in moderate and severe PWH. (**a**) Representative pictures of TRAcP staining of osteoclast cultures obtained by the differentiation of PBMC isolated from one adult moderate PWH (patient #1) and three age-matched healthy donors (HD). (**b**) Number of osteoclasts (OC) *per* field of (**a**). (**c**) Gene expression analysis of *RANK*, *TRAF6*, *CTSK* and *TCIRG1* in osteoclasts of adult patient with moderate haemophilia (patient #1) and healthy control. (**d**) Representative pictures of TRAcP staining of osteoclast cultures obtained by the differentiation of PBMC isolated from three HD and one pediatric severe patient before and after treatment with emicizumab. (**e**) Number of osteoclasts *per* field of (**d**). (**f**) Number of osteoclasts *per* field of ten HD and three adolescent patients with mild haemophilia. Results are expressed as mean ± SD.

A trend of increase of $CD16^{-}CD14^{++}$ cells was observed in both adult moderate (patient #1) and pediatric severe patients with no alteration of intermediate cells (Fig. 4a–c,e–g). Moreover, these patients showed a slight reduction of $CD16^{++}CD14^{+}$ population (Fig. 4a,d,e,h). Interestingly, the treatment of pediatric patients with FVIII mimicking antibodies highlighted a reduction of classical cells reaching levels observed in HD as well as a greater reduction of the non-classical subset, with no relevant modulations of the intermediate monocytes (Fig. 4e,h). No differences in monocyte sub-populations were observed in adolescent mild PWH (see Supplementary Fig. S3e–h on line). These data suggest that alterations of monocyte subsets may be directly involved in the increased osteoclast formation observed in cultures of PBMC isolated from patients with moderate/severe haemophilia.

In vitro effects of coagulation factors on osteoblasts

To investigate the involvement of osteoblasts in reduced BMD associated with haemophilia, we differentiated bone marrow mesenchymal stem cells into osteoblasts as shown by their expression of osteoblast markers including runt-related transcription factor 2 (*RUNX2*), osterix (*SP7*), alkaline phosphatase (*ALP*), collagen type I alpha 2 chain (*COL1A2*), Bone sialoprotein (*BSP*) and osteocalcin (*BGLAP*) (see Supplementary Fig. S4 online). We treated osteoblasts with coagulation factors. As shown in Fig. 5, treatment of human mature osteoblasts for 48 h with FVIII and VWF reduced cell positivity to ALP staining compared to PBS-treated cells (Fig. 5a,b). FIXa and FXa treatment did not affect ALP staining also considering different concentrations (see Supplementary Fig. S5a–d online). Interestingly, 50 nM thrombin was able to increase ALP positivity and no additional effect was observed at higher concentration (100 nM) (Fig. 5c,d). To evaluate the effect of the treatments on the mineralization activity of osteoblasts, we treated mature osteoblasts for 2 months with the coagulation factors. As shown in Fig. 5e,f, Alizarin red staining revealed a reduction of mineralized nodules following treatment with FVIII, VWF and FVIII/VWF, while no alterations were observed with FIXa, FXa and THB. Unfortunately, we could not characterize patients' osteoblasts since a bone biopsy is needed, but it is not required in the routine clinical management of PWH.

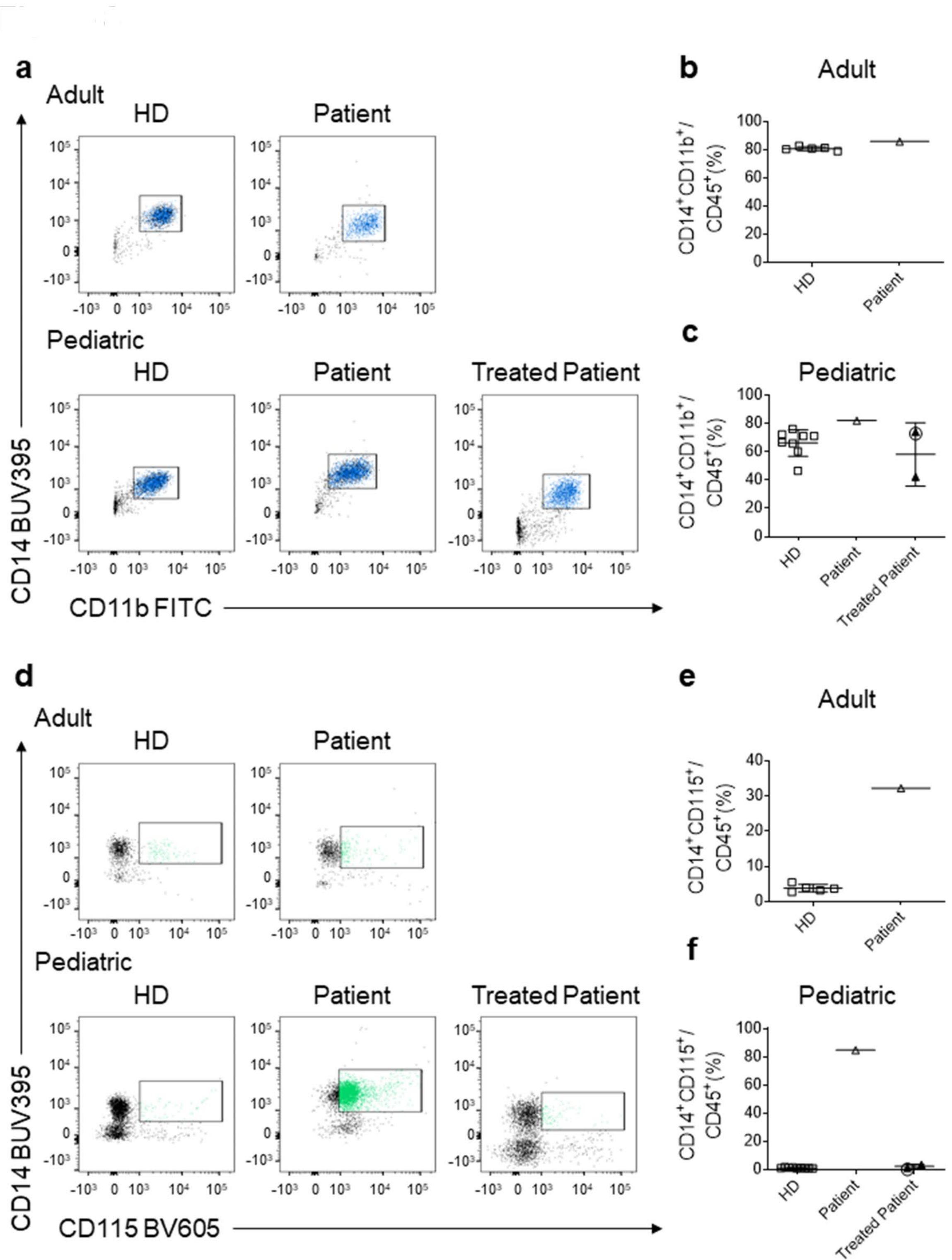

Fig. 3. Evaluation of osteoclast precursors in monocytes $CD45^+$ cell population of adult and pediatric patients with haemophilia. (**a**) Representative FACS plots of $CD14^+CD11b^+$ monocytes in the gate of $CD45^+$ cells in adult moderate (*upper panels*) and pediatric severe untreated or treated patients with haemophilia (*lower panels*) and age-matched HD. (**b**,**c**) Percentage of $CD14^+CD11b^+$ cells in the gate of $CD45^+$ population, in (**b**) adult moderate, and (**c**) pediatric severe patients. (**d**) Representative FACS plots of $CD14^+CD115^+$ population in the monocyte gate of adult moderate (*upper panels*) and pediatric severe patients (*lower panels*). (**e**,**f**) Percentage of $CD14^+CD115^+$ in the gate of $CD45^+$ cells, in (**e**) adult moderate and (**f**) pediatric severe patients. Adult moderate: patient #1, Table 1; pediatric severe patients: patients #2 and #6, Table 1. In (**c**) and (**f**) empty and filled triangles indicate patient #2 before and after emicizumab treatment, respectively; circled triangle represents patient #6 treated with denecimig. Results are expressed as mean ± SD.

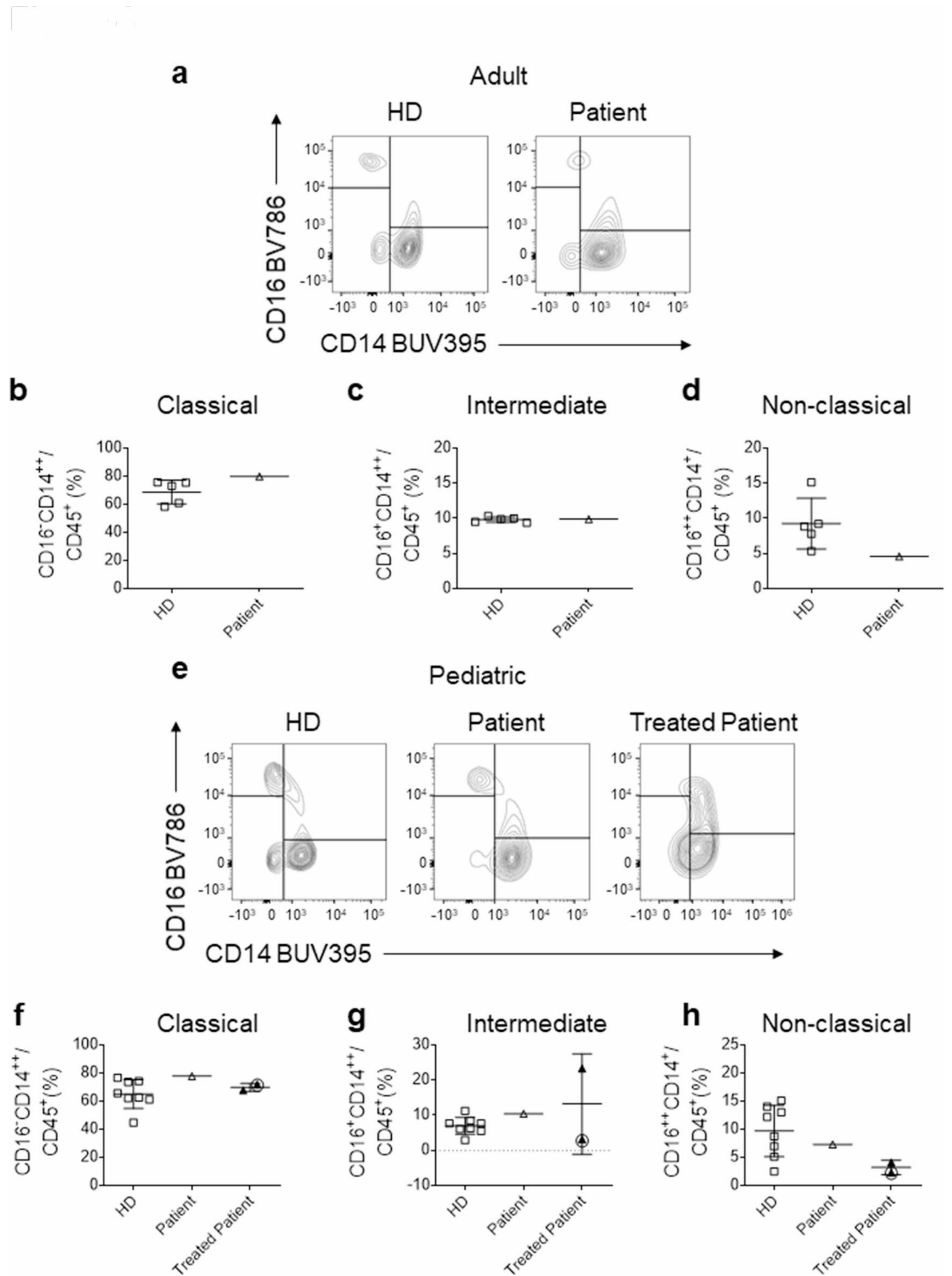

Fig. 4. Monocyte subsets distribution in PWH. (**a**,**e**) FACS plots showing the monocytes subpopulations in adult moderate and pediatric severe untreated or treated patients and age-matched healthy donors (HD). In monocyte gate, (**b**,**f**) the classical ($CD16^{-}CD14^{++}$), (**c**,**g**) the intermediate ($CD16^{+}CD14^{++}$), and (**d**,**h**) the non-classical ($CD16^{++}CD14^{+}$) populations were identified. Adult moderate: patient #1, Table 1; pediatric severe patients: patients #2 and #6, Table 1. In (**f**–**h**) empty and filled triangles indicate patient #2 before and after emicizumab treatment, respectively; circled triangle represents patient #6 treated with denecimig. Results are expressed as mean ± SD.

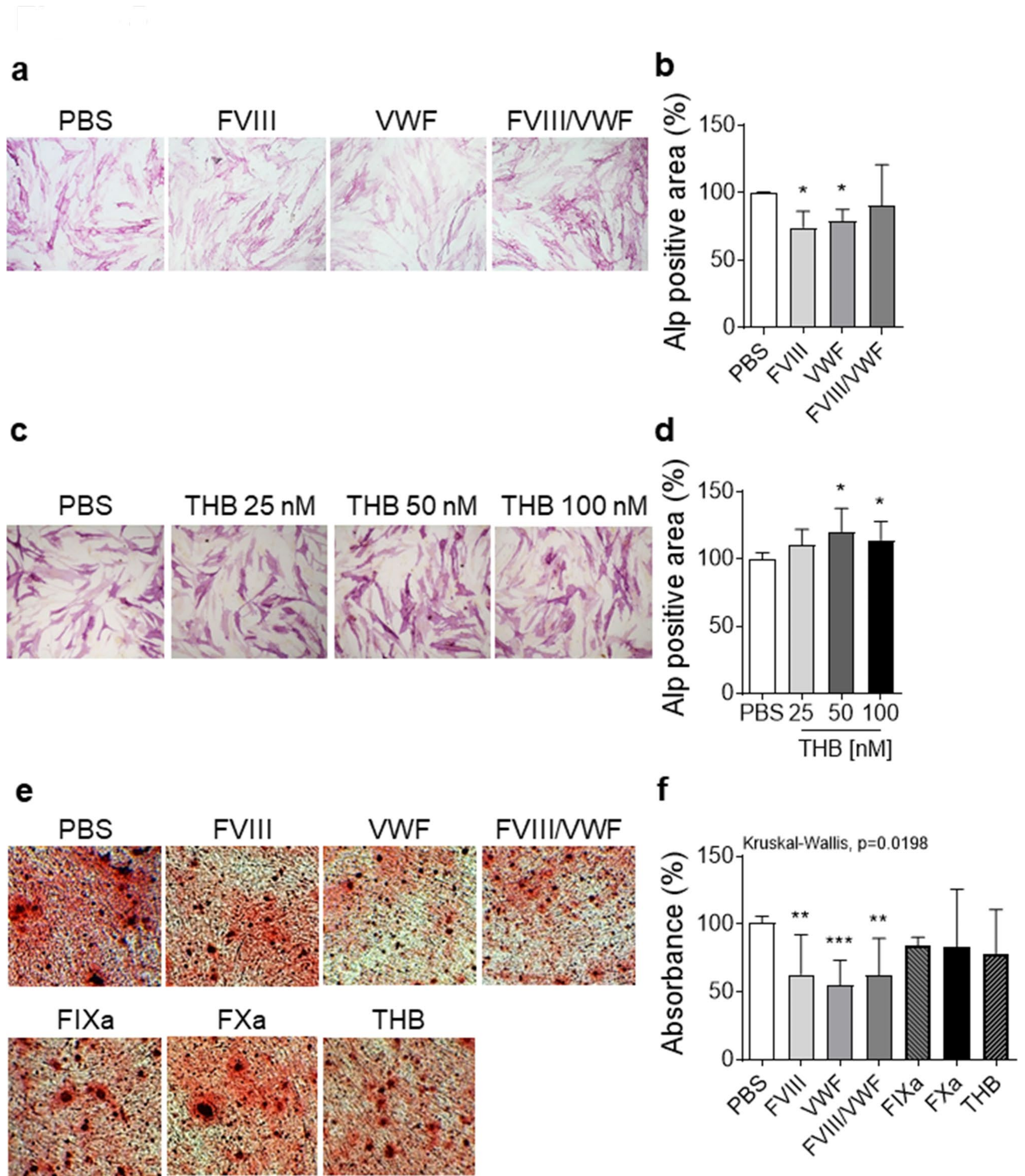

Fig. 5. In vitro effects of coagulation factors on osteoblasts. Human control osteoblasts were cultured for 48 h with FVIII (2 U/ml), VWF (20 μg/ml), FVIII/VWF complex (2 U/ml) and THB. (**a**) Representative pictures of ALP positive osteoblasts. Original magnification: 10×. (**b**) Densitometric analysis of ALP positive staining of osteoblasts. (**c**) Representative pictures of ALP positive osteoblasts treated with different concentrations of THB. (**d**) Densitometric analysis of ALP staining. Results are expressed as a percentage of ALP-positive area in cultures of coagulation factors-treated osteoblasts vs. PBS-treated cells and as mean ± SD. (**e**) Alizarin red staining of osteoblast treated with FVIII (2 U/ml), VWF (20 μg/ml), FVIII/VWF complex (2 U/ml), FIXa (10 nM), FXa (10 nM), THB (50 nM) or PBS. Original magnification: 4×. (**f**) Absorbance analysis of Alizarin red staining. Results are expressed as a percentage of absorbance of coagulation factors-treated cultures vs. PBS-treated cells and as mean ± SD. p-values are reported as each treatment compared to PBS. $^*p \leq 0.05$, $^{**}p \leq 0.01$ and $^{***}p \leq 0.001$.

Discussion

To better understand the mechanisms of bone alterations in PWH we investigated in vitro the direct effects of coagulation factors on bone cells. This experimental approach showed that FVIII, VWF, FVIII/VWF complex, FXa, and thrombin can inhibit osteoclastogenesis. Interestingly, in our in vitro experiments FIXa did not affect osteoclast differentiation. This is in contrast with the alterations of bone phenotype observed by Taves et al. in animal models; indeed, $FVIII^{-/-}$ and $FIX^{-/-}$ mice, but not $VWF^{-/-}$ animals, display an osteoporotic phenotype characterized by reduced bone mineral density[39]. However, the direct effects of FVIII and FIX deficiency on bone cells have not been deeply investigated; indeed, the increased osteoclast number in $FVIII^{-/-}$ and $FIX^{-/-}$ mice with knee haemarthrosis, was mainly related to the alteration of RANK-L/OPG ratio and the increase of IL-6 that our group demonstrated having detrimental effects on the skeleton[40].

In the present study, we also demonstrated that VWF was able to reduce osteoclastogenesis and bone resorption activity. Taves and co-authors have already hypothesized that $VWF^{-/-}$ mice would exhibit alteration of bone density, particularly following injury[39]. Moreover, population studies suggest that both male and female patients with von Willebrand disease showed an increased risk of osteoporosis and fractures in a large data base analysis[41]. Indeed, both female and male patients with von Willebrand diseases showed higher prevalence of osteoporosis (RR: 1.8 and 3.6 for female and male cases, respectively) and bone fracture (RR: 2.0 and 2.1 for female and male subjects, respectively) than healthy controls[41]. Interestingly, we recently demonstrated that circulating extracellular vesicles isolated from not-haemophilic patients with osteoporosis did not express von Willebrand factor[42]. These studies increase the relevance to investigate the effect of VWF on bone remodelling activity.

The majority of studies regarding the effects of coagulation factor deficiency on bone cells involved animal models. In the present study, we also performed in vitro experiments using PBMC isolated from PWH. We observed that PBMC isolated from untreated moderate and severe patients are characterized by an increased ability to differentiate into osteoclasts, due to the high prevalence of osteoclast precursor populations. In fact, an increase of $CD14^{+}CD115^{+}$ cell subset was detected in patients with severe haemophilia compared to healthy donors. CD115 is the receptor of colony stimulating factor 1 (CSF1R), also known as M-CSFR[43]. The interaction between M-CSF and its receptor is essential for osteoclastogenesis, since it stimulates the proliferation and expression of RANK receptor in osteoclast precursors[43]. To better investigate the osteoclast progenitors, we also studied the three different classes of monocytes: classical, intermediate and non-classical populations. Indeed, Sprangers et al. demonstrated that the classical monocytes in the blood are the primary osteoclast precursor cells and that in inflammatory conditions the intermediate monocytes could differentiate into osteoclasts[44]. Moreover, in vitro experiments demonstrated that non-classical cells are able to form multinucleated cells with osteoclast-like appearance but not able to resorb bone[44]. We observed a slight increase of $CD16^{-}CD14^{++}$ monocytes that are more prone to differentiate into osteoclasts than $CD16^{+}CD14^{+}$ cells, according to Komano et al.[37]. and Xue et al.[45]. However, further studies are needed to deeply clarify the roles of non-classical and intermediate monocytes in osteoclastogenesis and in the pathogenesis of bone alterations occurring in haemophilia. Indeed, it has been suggested that osteoclast formation in physiology and pathology is regulated by several pathways under the influence of different mechanisms or mediators[44]. We also evaluated osteoclasts and their precursors in pediatric patients treated with emicizumab and denecimig, bispecific factor IXa- and factor X-directed antibodies designed to facilitate their molecular recognition, thus able to restore the blood clotting process in PWH[46]. Interestingly, we observed a reduction of osteoclast differentiation of PBMC isolated from the patient after prophylaxis with emicizumab. This effect was probably due to the reduced percentage of $CD14^{+}CD11b^{+}$ cells and particularly of $CD14^{+}CD115^{+}$ population, showing similar levels to those observed in HD. Interestingly, in the other pediatric patient treated with denecimig, we found levels of these cell populations similar to those observed in emicizumab treated patient. Unfortunately, we had not enough cells to perform osteoclastogenesis assay and we had not samples of the same patient before starting denecimig therapy to evaluate modulations of osteoclast precursors before and after the treatment. These findings can only suggest but not definitively demonstrate a positive role of FVIII-mimetics on bone health in haemophilia patients. Both emicizumab and denecimig have very different molecular structures from FVIII: thus, we can hypothesize that the observed effects arise mostly from the enhancement of FXa and thrombin generation. Likewise, in vitro experiments showing the null effect of FIXa per se on osteoclast differentiation represents an implicit evidence that a reduced bone mineral density also observed in patients/mice with severe hemophilia B[39,47] would stem from a reduced FXa and thrombin generation.

Anagnostis et al. described haemophilia as a high bone turnover disease due to increased osteoclast activity and, in turn, enhancement of osteoblast response, as demonstrated by an increased bone-ALP in patients with low BMD[48]. Moreover, FVIII total knockout mice showed bone loss associated with increased bone resorption in female animals and a decline in bone formation in male mice[49]. The findings obtained in the present study not only confirm the results of animal studies[49] but show how direct effects of FVIII/VWF, thrombin, and FXa favor osteoclast proliferation and bone resorption. These results could have a possible impact on the broad therapeutic panel for hemophilia patients. Indeed, our results underlined the inhibitory effects of FVIII, VWF, VWF/FVIII, FXa and Thrombin on osteoclast differentiation suggesting that, in addition to preventing bleeding, replacing drugs could be also useful to prevent or treat bone loss, joint damage including the bone erosion associated with chronic synovitis. Moreover, inhibitors of osteoclasts proliferation could be tested in controlled clinical trials in association with replacing and also not-replacing drugs. For instance, Denosumab, a fully human monoclonal anti-RANK-L antibody that specifically inhibits the interaction between RANK-L and RANK, and strongly suppresses osteoclast differentiation and bone resorption[50], could be a valuable drug to prevent osteopenia, osteoporosis progression and chronic synovitis associated-bone erosion in haemophilia patients. To verify this possible pharmacological strategy, extensive case-controlled studies are needed.

We also evaluated the effects of coagulation factors on osteoblasts, showing that FVIII and VWF treatments reduce ALP positivity. Regarding the ability to form mineralized nodules, we observed that FVIII, VWF and FVIII/VWF decreased the mineralization activity of control osteoblasts. The inhibitory effect of these factors on bone mineralization and osteoclasts could explain the high bone remodeling activity observed in some PWH leading to the reduced bone mass[48]. FIXa and FXa treatment did not affect alkaline phosphatase and mineralization activity of mature osteoblasts. The lack of effects on bone cells following FIXa treatment suggests that the reduced BMD observed in patients with hemophilia B or in animal model, could be due to the alterations of multiple cytokines that are able to influence the bone remodeling activity and to thrombin generation. We showed that the treatment with 50 nM thrombin increases ALP activity, that could be in agreement with a previous study showing the ability of thrombin to stimulate proliferation and inhibit apoptosis of osteoblasts[51,52]. However, we did not observe any modulations of the mineralization activity of the thrombin-treated osteoblasts. Although osteoblasts express thrombin receptors[53], further studies are needed to dissect the molecular mechanisms underlining the effects observed in vitro. From the findings obtained in this study we can say that the studied coagulation factors affect the osteclastogenesis pathway and can influence osteoblasts, raising important questions regarding the mechanisms responsible for these effects. It has been demonstrated that thrombin and FXa have specific receptors on monocyte progenitors, which are represented by the protease-activated-receptors 1–2 (PARs 1–2); it is likely that the observed effects on osteoclasts by FXa and thrombin may derive from the interaction of these enzymes with these membrane receptors[33,54]. Regarding the effect of VWF and FVIII/VWF complex on osteoclasts, it has been demonstrated that VWF can bind LPR1 (low-density lipoprotein receptor-related protein-1), a large transmembrane protein involved in the clearance of lipoproteins, protease-protease-inhibitor complexes, and the FVIII/VWF complex[55–58]. Indeed, the interaction of FVIII/VWF complex with LRP1 plays a specific role in triggering pro-inflammatory signaling in macrophages and mediates osteoblast proliferation and activity. Interestingly, knockdown of LRP1 in murine RAW 264.7 cells inhibits osteoclast differentiation[59].

In conclusion, the present hypothesis-generating study revealed by in vitro experiments and even by analysis of several ex vivo measurements, that coagulation factors (FVIII, VWF, FVIII/VWF, FXa and thrombin) can directly and cooperatively affect bone cells and bone remodeling. However, a limit of the study could be related to the in vitro approach used to test the effects of single coagulation factor on bone cells; indeed, in vivo coagulation factors never act alone since they are part of the coagulation system and could be also in competition with other proteins that are not included in our experimental approach. Moreover, our ex vivo findings should be interpreted with caution, since they derived from the analysis of cells isolated from patients with different severities and treatments. A further multicentric study should be performed to recruit a larger cohort of patients to provide a deeper understanding of the relationship between the altered bone remodeling activity and both haemophilia type and severity.

Methods

Patients

Six patients and 21 male Healthy Donors (HD) were recruited by the Department of Translational Medicine and Surgery, Catholic University of the Sacred Heart, Policlinico Universitario "A Gemelli", IRCCS, Rome, Italy, and by the Pediatric Hematology/Oncology Department, Bambino Gesù Children's Hospital, IRCCS, Rome, Italy). For experiments involving human participants, informed consent was obtained from all subjects and/or their legal guardians, in accordance with the Declaration of Helsinki and local ethical approval (see below for details). The six haemophilia A patients were all negative for viral infections (HIV, HCV, and HBV). The age of HA patients ranged from 15 months to 43 year., while the severity of the disease was from mild to severe; the anti-haemophilia therapy was represented by both on-demand and prophylactic type, being the drugs represented by both replacing and non-replacing therapies. The analytical description of patients is reported in Table 1. They did not present any clinical signs of acute inflammation as revealed by normal value of C-reactive proteins (CRP < 0.5 mg/dL). Age-matched healthy donors (HD, $n = 5$: 16-month-old; $n = 3$: range 5–9 years; $n = 8$: range 14–19 years; $n = 5$: range 40–43 years) were free of clinical disorders as assessed by medical history. They were carefully checked for being free of any clinical and echographic signs of arthropathy, FVIII/VWF levels in the normal range, blood chemistries and blood cell count (C-reactive protein, LDH, white blood count, Hb level, creatinine, transaminases) to exclude any inflammatory status or organ damages. No PWH nor any HD smoked.

Osteoclast cultures

To test the effect of coagulation factors on osteoclastogenesis, HD-derived Peripheral Blood Mononuclear Cells (HD-PBMC) were isolated from EDTA-blood samples diluted in Phosphate Buffered Saline (PBS) solution and layered over Ficoll 1.077 g/ml (Lympholyte, Euroclone, Italy). After centrifugation at 400 g for 30 minutes, "buffy coat" was then collected, washed twice with PBS and resuspended in DMEM (Dulbecco's Modified Eagle Medium) medium containing 50 U/ml penicillin, 50 mg/ml streptomycin, 2 mM L-glutamine and 10% Fetal Bovine Serum (GIBCO, US). Then 8×10^5 cells/cm^2 were plated on cell culture dishes. After 3 hours, cell cultures were rinsed to remove non-adherent cells. Adherent cells were induced to differentiate into mature osteoclasts in presence of 20 ng/ml M-CSF (PeproTech, UK) and 30 ng/ml RANK-L (PeproTech, UK). Medium also contained PBS or OPG (25 ng/ml), human recombinant FVIII (Octocog alfa, 2 U/ml), human recombinant VWF (a generous gift by Takeda, 20 μg/ml), human FVIII/VWF complex (Fanhdi®, Grifols, Italy, 2 U/ml), human FIXa (Enzyme Research Laboratories, IN, USA, 10, 20, and 40 nM), human FXa (Merck, Italy, 10, 20, and 40 nM) and human thrombin (Enzyme Research, UK, 25, 50, and 100 nM). The evaluation of the effects of FIXa, FXa and thrombin was carried out over a nanomolar concentration range, comparable to that generated upon triggering the coagulation cascade in vitro[60,61]. On the other hand, the fixed concentrations of FVIII, VWF, and FVIII/VWF complex (≅ 200 U/dl) were chosen in the upper limit of the physiological range

to saturate the system and better evaluate the effect of these factors, as also shown in previous studies on the interaction between VWF and macrophage LRP1[62] and VWF-induced macrophage M1 polarization[63]. Medium with factors was replaced every 3–4 days for 14 days; cells were then fixed and stained for osteoclast marker TRAcP (Sigma-Aldrich, USA). DAPI staining was performed to identify multinucleated (≥ 3 nuclei) cells. The effect of coagulation factors on osteoclastogenesis was evaluated counting TRAcP-positive multinucleated (≥ 3 nuclei) cells and the number of nuclei/cells. To evaluate the osteoclastogenic potential of PBMC isolated from patients, cells were induced to differentiate into mature osteoclasts with osteoclastogenic cytokines M-CSF and RANK-L for 14 days. Regarding the two recruited pediatric patients (#2 and #6), we had enough cells to perform osteoclastogenesis assay for the patient #2 only.

Bone resorption

PBMC were cultured in 96 well multiplates containing 4 × 4 mm bovine bone slices (IDS, United Kingdom) and differentiated into osteoclasts as described above. Mature osteoclasts were then treated for 4 days with coagulation factors and cells were eventually fixed in paraformaldehyde; slices were cleaned free of cells by prolonged sonication, stained with 1% toluidine blue and observed by conventional light microscopy. Resorption pit area was measured by image analysis system (NIS Elements BR 4.50.00).

RNA extraction, reverse transcription and RT-qPCR

Total RNA was extracted from cells by using TriReagent (Sigma-Aldrich, USA) following the manufacturer's protocol. The concentration of RNA was determined by 260/280 nm absorbance using a NanoDrop ND-1000 spectrophotometer (ThermoFisher Scientific, USA), and the integrity of RNA was checked using gel electrophoresis. Total RNA (1 µg) was reverse-transcribed by using SensiFAST cDNA synthesis kit (Bioline, UK). RT-qPCR analysis was performed using SensiFAST SYBR Lo-ROX (Bioline, UK) according to the manufacturer's instructions. Primers for the gene being analyzed and for the housekeeping gene *GAPDH* are listed in Table 2. Each sample was analyzed in triplicate. The $2^{-\Delta\Delta ct}$ method was used for relative quantitation of gene expression, and results are expressed as $\log_{10}(2^{-\Delta\Delta ct})$.

Flow cytometry analysis

To evaluate circulating osteoclast precursors, 1×10^6 PBMC were incubated in the dark for 20 min at 4 °C with 1:40 directly conjugated monoclonal antibodies (Becton Dickinson, USA) against the following human surface molecules: CD11b (FITC conjugated), CD14 (BUV395 conjugated), CD16 (BV786 conjugated), CD45 (BUV 805 conjugated) and CD115 (BV605 conjugated). After labeling, cells were washed twice in PBS/FBS 2%, and data were acquired by FACS LSRFortessa (Becton Dickinson, USA). Flow cytometer profiles were analyzed using FlowJo™ v10.8 software (Becton Dickinson, USA) and the gating strategy is reported in Supplementary Fig. S2 online.

Osteoblast cultures

Osteoblasts were obtained from the differentiation of human Mesenchymal Stem Cells (MSC) isolated from bone marrow (Lonza Group AG, Switzerland). Briefly, MSC treated for three weeks with 2 mM β-glycerophosphate (Sigma-Aldrich, USA), 50 µg/ml ascorbic acid (Sigma-Aldrich, USA) and 10^{-8} M Dexamethasone (Sigma-Aldrich, USA). Cells were tested for ALP activity (Sigma-Aldrich, USA) and for the gene expression of osteoblast markers (Runx2, Sp7, Alp, Col1a2, Bsp and Bglap). Then, osteoblasts were treated with coagulation factors as following: FVIII (2 U/ml), VWF (20 µg/ml), FVIII/VWF complex (2 U/ml), FIXa (10, 20 and 40 nM), FXa (10, 20 and 40 nM), THB (25, 50 and 100 nM) or PBS. After 48 h of culture, ALP staining was performed according to the manufacturer's instructions.

Mineralization assay

Osteoblasts were cultured in DMEM + FBS supplemented with 2 mM β-glycerophosphate, 50 µg/ml ascorbic acid and 10^{-8} M Dexamethasone, and coagulation factors as following: FVIII (2 U/ml), VWF (20 µg/ml), FVIII/VWF complex (2 U/ml), FIXa (10 nM), FXa (10 nM), THB (50 nM) or PBS. Medium and factors were

Gene	Forward	Reverse
RANK	5′-CTGTTCCTCACTGAGCCTGG-3′	5′-TGCTGGCTTCTTCTTCAGGG-3′
TRAF6	5′-GCGTTATACCCGACTCTGGG-3′	5′-GCATGCACAGTTTGTACCCG3′
CTSK	5′-CCCGGAGTAATGACACCCTT-3′	5′-TCGGGGATCTCTCTGTACCC-3′
TCIRG1	5′-CCGCTCCCTACACCATCATC-3′	5′-GGGATCCAGGGTAAGCATCG-3′
RUNX2	5′-TTACTTACACCCCGCCAGTC-3′	5′-TATGGAGTGCTGCTGGTCTG-3′
SP7	5′-TGCTTGAGGAGGAAGTTCAC-3′	5′-AGGTCACTGCCCACAGAGTA-3′
ALP	5′-GGACATGCAGTACGAGCTGA-3′	5′-CCACCAAATGTGAAGACGTG-3′
COLIA2	5′-TGTGGATACGCGGACTTTGT-3′	5′-CAGCAAAGTTCCCACCGAGA-3′
BSP	5′-CAAGGGCACCTEGAAGACAA-3′	5′-CTCGGTAATTGTCCCCACGA-3′
BGLAP	5′-GACTGTGACGAGTTGGCTGA-3′	5′-CTGGAGAGGAGCAGAACTGG-3′
GAPDH	5′-GGATTTGGTCGTATTGGG-3′	5′-GGAAGATGGTGATGGGATT-3′

Table 2. Sequence of primers.

replaced every 3–4 days. After two months, cultures were washed with PBS and fixed with 70% cold ethanol for 1 h. Mineralized nodules were stained with 0.1% alizarin red solution. For quantification analysis, alizarin red staining was dissolved with 10% cetylpyridinium chloride in 10 mM sodium phosphate (pH 7.0) and the absorbance was measured in the microplate reader at 562 nm.

Statistical analyses

Data are expressed as the mean ± SD and at least three independent experiments were performed to study the effects of coagulation factors on HD bone cells. Statistical analysis was performed by one-way analysis of variance, followed by the Mann-Whitney U test. If needed, the comparison between groups was carried out by using the Kruskal-Wallis test. A p-value ≤ 0.05 was considered statistically significant.

Study approval

This study was approved after a full ethical review by the Institutional Review Board (IRB) of the Fondazione Policlinico Universitario "A. Gemelli" IRCCS (Lazio Roma 3, approval #22082) and IRB of the Bambino Gesù Children's Hospital (approval #2426_OPBG_2021). The data were accessed anonymously.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Author contributions

G.B., M.S., M.R., S.T. and Laura D.G. performed in vitro experiments and FACS analysis; J.D.G., M.D.A., O.P., M.T., Leonardo D.G., S.P., S.M. and R.M.T. were involved in the acquisition and analysis of data; R.D.C. and M.L. were involved in the recruitment of patients. S.L., A.D.F., M.L. and R.D.C. were responsible of the conception and design of the study, interpretation and analysis of data and drafting the article. All authors revised the manuscript and approved the submitted version.

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Declarations

Competing interests

The authors declare no competing interests.

Institutional review board approval

This study was approved after a full ethical review by the Institutional Review Board (IRB) of the Fondazione Policlinico Universitario "A. Gemelli" IRCCS (Lazio Roma 3, approval #22082) and IRB of the Bambino Gesù Children's Hospital (approval #2426_OPBG_2021). The data were accessed anonymously.

Additional information

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-024-75747-w.

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