Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. (A) Pipeline for profiling of RBC miRNAs during storage. Leukodepleted RBC units from three different individuals were incubated at 4 °C for the indicated number of days. Aliquots of RBCs were removed from the bag for RNA isolation with the addition of nonhuman miRNAs for normalization on the NanoString platform. (B) Select upregulated and downregulated miRNAs are featured; non-colored lines refer to most microRNAs without significant changes. (C) A separate line plot for miR-720 with mean ± SD. (D) Ethidium bromide stain of the northern blots of RBCs during different dates of storage. (E) Cloverleaf structure of $tRNA^{Thr(TGT)}$ and miR-720 (red) probe are illustrated. Figures were modified from Schopman *et al.* (F) Small RNA northern blot with miR-720 probe detecting RNA from RBCs stored for 4 and 28 days together with synthetic 18mer miR-720 as size markers. (G) Cloverleaf structure of $tRNA^{Thr(TGT)}$ and full-length probe (blue) are illustrated. (H-I) Northern blots of the RBC RNA stored for different days probed with (H) tRNA- $^{Ala(GCA)}$ or (I) tRNA- $^{Tyr(UAA)}$ full-length probes.

Figure S2. (A) Heat inactivation (HI) abolishes the cleavage activity in RBC lysates. Northern blot data show heat inactivation from two different donors with miR-720 probe. (B) Dicer, ANG and ANG inhibitor RNH1 are present in RBCs. Western blots of the indicated proteins in RBC lysate during storage. Ponceau S staining and 4.1R serve as loading controls. (C) Synthetic $tRNA^{Thr(TGT)}$ was incubated with indicated proteins (DNase I, RNase H and ANG) and detected by northern blot with the miR-720 probe.

Table SI. The sequence(s) of the synthetic RNAs and northern blot probe(s).

Appendix S1. Supplemental methods.

References

Bryk, A.H. & Wisniewski, J.R. (2017) Quantitative analysis of human red blood cell proteome. *Journal of Proteome Research*, **16**, 2752–2761.

Chen, P.-H., Hong, J. & Chi, J.-T. (2017) Discovery, genomic analysis, and functional role of the erythrocyte RNAs. *Current Pathobiology Reports*, **5**, 43–48.

D'Alessandro, A., Kriebardis, A.G., Rinalducci, S., Antonelou, M.H., Hansen, K.C., Papassideri, I.S. & Zolla, L. (2015) An update on red blood cell storage lesions, as gleaned through biochemistry and omics technologies. *Transfusion*, **55**, 205–219.

Kannan, M. & Atreya, C. (2010) Differential profiling of human red blood cells during storage for 52 selected microRNAs. *Transfusion*, **50**, 1581–1588.

Li, L.Z., Zhang, C.Z., Liu, L.L., Yi, C., Lu, S.X., Zhou, X., Zhang, Z.J., Peng, Y.H., Yang, Y.Z. & Yun, J.P. (2014) miR-720 inhibits tumor invasion and migration in breast cancer by targeting TWIST1. *Carcinogenesis*, **35**, 469–478.

Sangokoya, C., Telen, M.J. & Chi, J.T. (2010) microRNA miR-144 modulates oxidative stress tolerance and associates with anemia severity in sickle cell disease. *Blood*, **116**, 4338–4348.

Sarachana, T., Kulkarni, S. & Atreya, C.D. (2015) Evaluation of small noncoding RNAs in ex vivo stored human mature red blood cells: changes in noncoding RNA levels correlate with storage lesion events. *Transfusion*, **55**, 2672–2683.

Schopman, N.C., Heynen, S., Haasnoot, J. & Berkhout, B. (2010) A miRNA-tRNA mix-up: tRNA origin of proposed miRNA. *RNA Biology*, **7**, 573–576.

Walzer, K.A. & Chi, J.T. (2017) Trans-kingdom small RNA transfer during host-pathogen interactions: the case of P. falciparum and erythrocytes. *RNA Biology*, **14**, 442–449.

Wang, Y., Zhang, Z., Ji, D., Chen, G.F., Feng, X., Gong, L.L., Guo, J., Li, Z.W., Chen, C.F., Zhao, B.B., Li, Z.G., Li, Q.J., Yan, H.P., Sempowski, G., Wang, F.S. & He, Y.W. (2015) Regulation of T cell function by microRNA-720. *Scientific Reports*, **5**, 12159.

Bleeding risk for patients with haemophilia under antithrombotic therapy. Results of the French multicentric study ERHEA

Haemophilia A and B are haemorrhagic diseases occurring mostly in male subjects and are classified as mild (from 6 to 40 IU/dl of coagulation factor), moderate (1–5 IU/dl) and severe (<1 IU/dl). Treatments of the most severe forms rely on the substitution of factor VIII (FVIII) or factor IX (FIX) on demand, or as long-term prophylaxis (Fischer *et al*, 2002). Until recently, the life expectancy of patients with haemophilia (PWH) was significantly reduced (Larsson, 1985). However, recent progress in therapy has led to improved survival (Lovdahl *et al*, 2013) and PWH are now exposed to age-related disorders, such as cardiovascular diseases (CVD) (Foley *et al*, 2010). The treatment of CVD relies on antithrombotic therapies (ATT), such as anti-platelets and anticoagulants; however, the potential bleeding risk associated with these treatments in PWH is not known. Although current recommendations about CVD in PWH propose systematic substitutive prophylaxis when coagulation factor activity is ≤5 IU/dl, they are mainly based on expert opinions (Mannucci & Mauser-Bunschoten, 2010; Martin & Key, 2016). The aim of this study was to assess the bleeding risk in PWH receiving ATT.

This multicentric, open, non-interventional study, registered at ClinicalTrials.gov NCT03157154, compared the bleeding risk in PWH under ATT and in a control group of

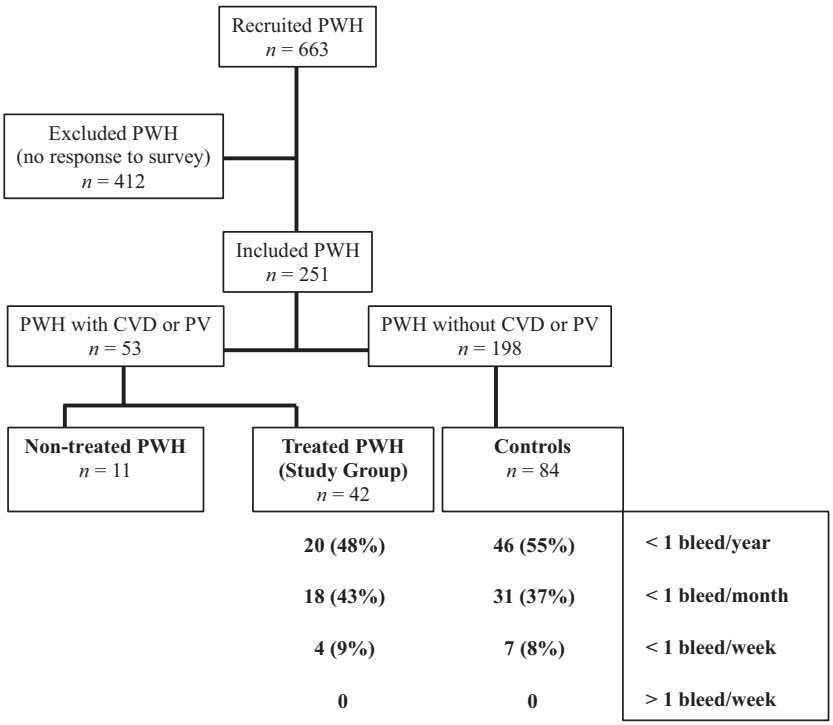

Fig 1. Flow chart and frequency of bleeding events in treated PWH or controls group. CVD, cardiovascular disease; PV, polycythaemia vera; PWH, patient with haemophilia.

PWH without ATT. PWH were matched on age (±5 years) and FVIII or FIX levels divided into 5 segments: <1, 1–5, 6–20, 21–35, 36–50 IU/dl). The PWH:controls ratio was 1:2. The primary objective was the overall bleeding risk evaluated within the 2 groups. The patients were separated into 4 subsets according to bleeding frequency: <1 event per year, <1 event per month, <1 event per week and ≥1 event per week. Secondary objectives were to investigate for (i) the presence of severe bleeds (SB) requiring substitutive treatment, hospitalization, transfusion or surgical/radiological intervention; (ii) risk factors for SB; (iii) prevalence of CVD and (iv) the impact of classical cardiovascular risk factors. Non-severe bleeds were defined as all bleeding events that did not meet severe bleeding criteria.

Inclusion criteria were: PWH, age ≥50 years, followed at least once in the past 5 years. All PWH received an information letter and a questionnaire allowing them to report clinical data during the past year. These data were then validated by consultation of their medical records. Patients were then separated in two groups, with or without a cardiovascular event. Among the patients with cardiovascular events, those undergoing ATT formed the study group and patients without CVD provided the matched control group. Unexpectedly, some PWH reported no CVD but anti-thrombotic treatment of polycythaemia vera (PV). Those were included in the study group.

Statistical analyses were performed using XLSTAT (Addinsoft, France) and considered statistically significant for *P*-values <0·05. The overall bleeding risk was analysed with Mann–Whitney and Friedman bilateral tests for univariate analysis and a logistical regression model for multivariate analysis. SB were analysed by χ^2 test. Risk factors for SB in the study group and impact of classical cardiovascular risk factors were analysed using a Fisher test in univariate analysis and a logistical regression model for multivariate analysis.

Of 663 patients recruited, 251 answered the survey (Fig 1). The prevalence of mild, moderate and severe haemophilia was similar between recruited patients and those included in the study ($P = 0{\cdot}33$). The median age of enrolled patients was 62 years (range 50–94). Fifty-one patients (20%) had a history of cardiovascular events and 2 received aspirin prophylaxis for PV (Table I). Among these 53 patients, 42 (79%) received an ATT (Table I). These 42 patients were matched on age and disease severity of the disease with the 84 controls. The bleeding risk was similar between these two groups ($P = 0{\cdot}49$ and $P = 0{\cdot}70$ for qualitative or quantitative tests). The same results were obtained in subgroup analysis for patients with mild haemophilia ($P = 0{\cdot}57$ and $P = 0{\cdot}29$) or on aspirin treatment ($P = 0{\cdot}32$ and $P = 0{\cdot}16$). SB were more frequent in the study group [odds ratio (OR) = 3·55, 95% confidence interval (CI) 1·2–10·4, $P = 0{\cdot}02$]. Although not statistically significant, a trend towards more frequent SB was also found in subgroup analysis for patients with mild haemophilia (OR = 2·16, $P = 0{\cdot}23$). The only risk factor for SB was the presence of previous non-SB [OR = 21, $P = 0{\cdot}001$ (univariate) and $P = 0{\cdot}038$ (multivariate)], which was higher when non-SB events were occurring more than once a month [OR = 169, $P < 0{\cdot}0001$ (univariate), $P = 0{\cdot}01$ (multivariate)]. No patient experienced SB without prior

Table I. Patients' characteristics.

Characteristic	Total	Haemophilia A	Haemophilia B
Severity of haemophilia			
Mild	176	155	21
Moderate	31	22	9
Severe	44	38	6
Medical events			
ACS/angina	20	16	4
Stroke	7	5	2
Stroke + PAOD	2	2	0
PAOD	5	5	0
PAOD + AF	2	2	0
AF	6	6	0
Valvulopathy	7	7	0
Aortic aneurysm	2	2	0
PV	2	2	0
Antithrombotic treatment			
Aspirin	29	26	3
Clopidogrel	4	4	0
Aspirin + VKA	2	2	0
VKA only	6	6	0
Heparin	0	0	0
DOA	1	0	1
Cardiovascular risk factors			
SHT	88	78	10
Dyslipidaemia	32	26	6
Obesity	24	16	8
Tobacco	44	35	9
Diabetes	16	13	3

ACS, acute coronary syndrome; AF, atrial fibrillation; DOA, direct oral anticoagulant (rivaroxaban); PAOD, peripheral arterial obstructive disease; PV, polycythaemia vera; SHT, systemic hypertension; VKA, vitamin K antagonist.

non-SB. The severity of haemophilia was not a risk factor for SB [$P = 0{\cdot}15$ (univariate); $P = 0{\cdot}84$ (multivariate)]. None of the 5 patients with moderate haemophilia initially received substitutive prophylaxis but 2 required prophylaxis during the ATT due to SB. All severe PWH ($N = 4$) were under long term prophylaxis and only one experienced a SB.

Statistically significant cardiovascular risk factors in this study were tobacco (OR: 3·4; 95% CI: 1·4–8·4, $P = 0{\cdot}009$), systemic hypertension (OR: 2·8; 95% CI: 1·2–6·5, $P = 0{\cdot}02$), dyslipidaemia (OR: 3·4; 95% CI: 1·2–9·3, $P = 0{\cdot}02$). Diabetes (OR: 0·7; 95% CI: 0·1–3·0, $P = 0{\cdot}60$) and obesity (OR: 1·2; 95% CI: 0·3–4·2, $P = 0{\cdot}82$) were not associated with CVDs.

The prevalence of CVDs in this study was 20%, similar to that reported previously (Rizwan *et al*, 2015). Likewise, most of the cardiovascular risk factors had a similar impact compared to the literature, except for the low prevalence of diabetes (6·4%) reported here. This study highlights that even if ATT is usually well tolerated in PWH, it can lead to increased bleeding, even in patients with mild haemophilia. The only risk factor found for SB was the presence, within the past year, of non-SB. All patients with severe haemophilia were under long-term prophylaxis before the initiation of ATT, which explains why haemophilia severity was not linked to SB.

Altogether, these results suggest that prevention and treatment strategies based only on deficient factor levels is insufficient to appreciate bleeding risks during ATT, especially in moderate and mild haemophilia, because patients with severe haemophilia are systematically on prophylaxis. A precise recording of non-SB by the patients, evaluated during medical consultations, might be an essential asset in risk assessment. In patients with mild haemophilia, this could be a valuable addition to classical therapeutic education tools, particularly as these patients are usually less involved in such programmes.

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This trial (ERHEA) was registered at https://clinicaltrials.gov/ct2/show/NCT03157154.

Author Contributions

AD and MT designed, performed and coordinated the research; collected, analysed and interpreted the data and wrote the manuscript. AD performed statistical analyses, produced the figures and edited the manuscript. AL, BG, PB, BPP, LA, FPV, MS, MF, CT and MCB included patients, contributed data and commented on the manuscript.

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References

Fischer, K., van der Bom, J.G., Molho, P., Negrier, C., Mauser-Bunschoten, E.P., Roosendaal, G., De Kleijn, P., Grobbee, D.E. & van den Berg, H.M. (2002) Prophylactic versus on-demand treatment strategies for severe haemophilia: a comparison of costs and long-term outcome. *Haemophilia*, **8**, 745–752.

Foley, C.J., Nichols, L., Jeong, K., Moore, C.G. & Ragni, M.V. (2010) Coronary atherosclerosis and cardiovascular mortality in hemophilia. *Journal of Thrombosis and Haemostasis*, **8**, 208–211.

Larsson, S.A. (1985) Life expectancy of Swedish haemophiliacs, 1831-1980. *British Journal of Haematology*, **59**, 593–602.

Lovdahl, S., Henriksson, K.M., Baghaei, F., Holmstrom, M., Nilsson, J.A., Berntorp, E. & Astermark, J. (2013) Incidence, mortality rates and causes of deaths in haemophilia patients in Sweden. *Haemophilia*, **19**, 362–369.

Mannucci, P.M. & Mauser-Bunschoten, E.P. (2010) Cardiovascular disease in haemophilia patients: a contemporary issue. *Haemophilia*, **16** (Suppl 3), 58–66.

Martin, K. & Key, N.S. (2016) How I treat patients with inherited bleeding disorders who need anticoagulant therapy. *Blood*, **128**, 178–184.

Rizwan, I., Minuk, L., Jackson, S. & Iorio, A. (2015) Cardiovascular disease prevalence and relevance in haemophilia: a scoping review. *Haemophilia*, **21**, e156–e166.

Genetic analysis of Diffuse Large B-cell Lymphoma occurring in cases with antecedent Waldenström Macroglobulinaemia reveals different patterns of clonal evolution

Waldenström Macroglobulinaemia (WM) is a rare indolent B-cell malignancy characterised by IgM paraprotein and bone marrow (BM) infiltration by clonal lymphocytes. The *MYD88* L265P mutation is seen in >90% patients with WM (Swerdlow *et al*, 2016). BTK inhibitors, such as ibrutinib, are recommended for treatment of WM (Castillo *et al*, 2016a; Talaulikar *et al*, 2017).

Diffuse large B-cell lymphoma (DLBCL) is known to occur in 2–10% of cases with antecedent WM (Lin *et al*, 2003; Leleu *et al*, 2009; Castillo *et al*, 2016b). It can result either from histological transformation of the WM clone or arise as a *de novo* lymphoma, with the latter presumably carrying a better prognosis.

We aimed to track the clonal relationship of DLBCL and antecedent WM in 4 cases with paired samples using *MYD88* L265P and immunoglobulin heavy chain variable region (*IGHV*) analysis.

The mean age of the 4 male patients was 71 years (range 61–89 years). Patient 1 received chlorambucil and prednisolone for WM followed by ibrutinib and rituximab at relapse; Patient 2 received dexamethasone and rituximab; Patient 3 was treatment-naïve at the time of DLBCL diagnosis and Patient 4 received fludarabine and cyclophosphamide. Patient 1 was on Ibrutinib at the time of diagnosis of DLBCL.

The average time to transformation in our cohort was 4·1 years (range 1·5–9), and patients were treated with high dose chemoimmunotherapy protocols +/− autologous stem cell transplantation and/or central nervous system (CNS) prophylaxis with high dose methotrexate.

WM was in partial remission in Patients 1 and 2 and in complete remission in Patient 4 at the time of transformation. The site of disease was extranodal in 3 of the 4 cases including the brain in Patient 1. Cell of origin was activated B cell subtype (ABC) in Patients 1 and 2 and germinal centre (GC) subtype in Patients 3 and 4 (Table SI). DLBCL occurring after antecedent WM is known to be of the ABC subtype in 80% of cases (Durot *et al*, 2017).

Formalin-fixed paraffin-embedded sections of paired diagnostic WM bone marrow trephines and subsequent histologically confirmed DLBCL were obtained for all 4 cases. Analysis was also performed on fresh bone marrow aspirate on Patient 1. DNA was extracted from all samples and used for *MYD88* L265P and immunoglobulin heavy chain (*IGHV*) polymerase chain reaction (PCR) and sequencing. The minimal amount of brain tissue from Patient 1 was subjected to laser-capture microdissection, *IGHV* PCR and cloning before sequencing (Appendix S1).

All 4 cases were heterozygous for the *MYD88* L265P mutation on diagnostic WM samples. Patients 1, 2 and 4 were also carried a heterozygous *MYD88* L265P mutation at time of transformation (see Figure S1). Occasional cases of *MYD88* L265P mutation occurring in transformed samples have been reported previously (Castillo *et al*, 2016b). Patients 2 and 3 had the same *IGHV* sequence as the initial WM sample, consistent with clonal evolution from the initial WM (Table I).

IGHV analysis on Patient 1 was limited by paucity of sample. Microdissection and sequencing on 10 selected clones showed the presence of *IGHV4-34*01* and *IGHV1-69*03* in 33% of clones, each with the overall result interpreted as polyclonal. Thus, the clonal origin of the DLBCL could not be conclusively confirmed.

MYD88 L265P analysis on the DLBCL sample from Patient 3 showed wild-type alleles, raising possibility of (i) synchronous lymphoma with a clonal origin independent to that of the pre-transformed WM or (ii) loss of *MYD88* L265P mutation during clonal evolution. *IGHV* analysis demonstrated a different *IGHV* clone to that of the initial