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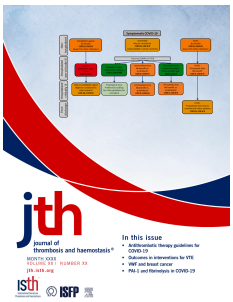

Little discrepancy between one-stage and chromogenic FVIII/IX assays in a large international cohort of persons with non-severe hemophilia A and B

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Little discrepancy between one-stage and chromogenic FVIII/IX assays in a large international cohort of persons with non-severe hemophilia A and B

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Essentials

- Factor VIII and IX activity can be measured by the one-stage and chromogenic assay
- Central measurements with both assays were performed in a large non-severe hemophilia cohort
- Over 90% of 220 persons had no discrepancy irrespective of the definition used
- Little discrepancy was found in persons with mutations previously associated with discrepancy

Abstract

Background: Accurate measurements of coagulation factor activity form an essential part of hemophilia management and are performed by the one-stage or chromogenic assay. Current literature suggests that approximately one-third of persons with non-severe hemophilia A exhibit assay discrepancy, although with a high variability between studies. Such data are scarce in non-severe hemophilia B.

Objective: To investigate the extent of FVIII/IX one-stage and chromogenic assay discrepancy in moderate and mild hemophilia A and B.

Methods: Persons with previously diagnosed non-severe hemophilia A and B with a factor level 2-35 IU/dL were included from the international DYNAMO cohort study. Central measurements of the FVIII and FIX activity levels were performed by the one-stage and chromogenic assay. Relative and absolute discrepancy definitions were used with the ISTH-SSC proposed ratio of >2.0 or <0.5 being the primary outcome. Discrepancy was also evaluated in a subgroup of 13 persons with mutations previously associated with discrepancy (≥3 cases reported in literature).

Results: A total of 220 persons were included of whom 3 (1%) showed assay discrepancy: 2/175 hemophilia A and 1/45 hemophilia B. Six persons (3%) exhibited an absolute difference >10 IU/dL between the assay results. Also with more lenient definitions over 90% of participants (n=197) had no discrepant results. Only 1 of 13 persons with a mutation previously associated with discrepancy had significant assay discrepancy.

Conclusion: Little assay discrepancy was observed despite the presence of mutations previously associated with discrepancy, suggesting that the presence and magnitude of assay discrepancy is largely determined by laboratory variables.

Keywords (MeSH): hemophilia A, hemophilia B, factor VIII, factor IX, blood coagulation tests

Introduction

Hemophilia A and B are rare inherited coagulation disorders that result from a deficiency in clotting factor VIII (FVIII) or factor IX (FIX), respectively. The diagnosis and management of these bleeding disorders are primarily based on the residual endogenous factor activity level that determines the classification as severe (<1 IU/dL), moderate (1-5 IU/dL) or mild (>5-40 IU/d)[1].

Different laboratory assays are used to measure FVIII and FIX activity. The one-stage assay (OSA) has been most widely adopted, with reported use in 90% of laboratories[2]. In this assay, patient plasma, plasma deficient in the coagulation factor of interest and reagents are mixed and the resulting activated partial thromboplastin time (APTT) is compared with a calibration curve from serial dilutions of reference plasma[3]. The chromogenic assay (CA) has been less frequently available as it is generally perceived as more expensive and technically complex[4,5] with a reported use in 68% of laboratories[2]. This assay measures the ability of FVIII or FIX to generate activated FX (FXa) and encompasses two stages. In the first stage, reagents are added to the patient plasma which leads to activation of FX. In the second stage, the amount of FXa produced is measured through its action on a chromogenic substrate. The colour intensity generated is proportional to the FVIII or FIX level that was present[3] as compared with a calibration curve from serial dilutions of reference plasma.

Current literature suggests that around one-third of persons with non-severe hemophilia A show discrepant results between the OSA and CA, although a high heterogeneity between studies is observed[6-8]. The Scientific and Standardization Committee (SSC) of the International Society on Thrombosis and Haemostasis (ISTH) defined a ratio of >2.0 or <0.5 as discrepant[9], although other studies have also used other definitions (i.e. based on absolute difference >10 IU/dL or ratio >1.5 or <0.6). Classically, the CA measures lower FVIII/IX activity levels than the OSA, but reverse discrepancy where the results of the OSA are lower than CA have also been reported[6,10]. Previous studies demonstrated a correlation between the presence of assay discrepancy and specific mutations in the *F8* gene, supporting the hypothesis for a genetic basis underlying assay discrepancy[7,11]. In general, lower levels by the CA compared to OSA were associated with missense mutations that result in a reduced stability of the FVIII protein[5,7]. In persons with lower levels by the OSA compared to the CA, mutations are frequently located at functional sites of the FVIII protein related to thrombin activation, binding to activated FIX (FIXa) or Von Willebrand Factor (VWF)[5,7]. In non-severe hemophilia B, knowledge on assay discrepancy is limited with a single study demonstrating discrepant findings in 25% of persons, all having lower FIX activity levels measured by OSA compared to CA[10].

Accurate measurement of FVIII and FIX activity levels form a critical part of hemophilia management, as the diagnosis and disease severity are established based on the residual endogenous factor level. In addition, factor levels are measured to guide treatment decisions and monitor therapy. To avoid potential misclassification, it has been recommended to perform both the OSA and CA in the diagnostic work-up of non-severe hemophilia A[7,9,12]. Knowledge on the extent of assay discrepancy and potential factors causing the heterogeneity observed is essential. As studies have reported varying results for hemophilia A and are scarce for hemophilia B, a large international study with centralized measurements may add more robust data to the existing body of work. Therefore, the aim of this study was to investigate the extent of assay discrepancy in persons with non-severe hemophilia A and B in a multicenter international setting. Additionally, assay discrepancy was assessed in a subgroup of persons with mutations associated with discrepancy in previous literature.

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Methods

Setting and design

The DYNAMO study was an observational cohort study that recruited persons with non-severe hemophilia among 15 hemophilia treatment centers from January 2018 to May 2021. Participating centers were located in the Netherlands, United Kingdom, Italy, Austria and Canada. Additional details on the participating sites can be found in the supplemental material. The study received approval from all institutional review boards and was registered at ClinicalTrials.gov (NCT0362395). Persons with non-severe hemophilia A or B with a baseline FVIII or FIX level between 2-35 IU/dL and aged 12-55 years were eligible for inclusion. As a consequence, the majority of this study cohort has been historically diagnosed with hemophilia based on results from a one-stage assay. Exclusion criteria were hemophilia B Leyden, history of a clinically relevant inhibitor, participation in a trial with an investigational product, use of anticoagulants and the presence of a comorbidity affecting the musculoskeletal system. All participants provided written informed consent for this study which included retrospective clinical data collection and a blood withdrawal. Data collected from medical files included demographics, *F8* or *F9* mutation according to the Human Genome Variation Society (HGVS) numbering and treatment regimen. All historical baseline factor levels measured locally were collected including assay type (one-stage, chromogenic). Definitions used for data collection are listed in the supplemental material.

Study outcome

The study outcome was discrepancy between baseline FVIII or FIX levels measured centrally by OSA and CA. The presence of assay discrepancy was defined in 2 ways: first, a ratio of >2.00 or <0.50 was defined as significant assay discrepancy as proposed by the SSC of the ISTH[9] and the second was defined as an absolute difference between assays of at least 10 IU/dL in accordance with previous work of Schütte et al[13]. The direction of assay results was reported as standard (higher OSA results) or reverse (higher CA results). For further detailed exploration, lenient assay discrepancy was defined as a relative difference between the assay results with ratios of >1.50 or <0.67 and an absolute difference between the assay results of at least 5 IU/dL. First, assay discrepancy was investigated in the full study population, according to different definitions of assay discrepancy. Then, a subanalysis was performed in a subgroup of persons carrying mutations associated with assay discrepancy in previous literature. Furthermore, centrally and locally measured levels by OSA and CA were compared in a subgroup of persons in whom factor activity levels were measured locally with both assays on the same day at one or multiple occasions.

Blood sampling and laboratory analysis

Blood withdrawal was performed during a clinic visit at the local hemophilia treatment center after a wash-out period of 3 days (hemophilia A) or 5 days (hemophilia B) for standard half-life products. For extended half-life products, the required wash-out period was determined by the treating physician. Blood was collected in citrated tubes and processed by the local laboratories. Samples were double-spun for 15 minutes at 3000g and 18°C. The obtained plasma was stored frozen at -80°C within 4 hours and shipped on dry ice to the coagulation laboratory of the Amsterdam UMC. After the transport, all samples were thawed once after which the measurements were performed. Measurements of FVIII and FIX activity levels were performed centrally by both OSA and CA. The FVIII and FIX one-stage assays were measured with Actin FS reagents from Siemens and the FVIII and FIX chromogenic assays were measured with kits from Siemens and Rossix, respectively. FVIII and FIX deficient plasmas were from Siemens in which other remaining coagulation factors, including VWF, were present in levels ≥40 IU/dL. The FVIII and FIX one-stage assays and FVIII chromogenic assay were calibrated using standard human plasma (SHP) from Siemens. The FIX chromogenic assay was calibrated using Biomed Hyphen Plasma Calibrator. Lot numbers are listed in the supplemental material. The chromogenic assays were based on end-point methodology. Different reference curves were used. In the chromogenic assay, FVIII samples >10 IU/dL and FIX samples >15 IU/dL were analyzed against a high reference standard curve (FVIII range: 136.5-1.4 IU/dL and FIX range: 194.0-12.1 IU/dL). FVIII samples <10 IU/dL and FIX samples <15 IU/dL were analyzed against a low reference standard curve (FVIII range: 11.4-0.4 IU/dL and FIX range: 24.3-0.4 IU/dL). In the one-stage assays, samples were analyzed against one standard reference curve in which specific low-range dilutions were used for samples <10 IU/dL. The incubation times were 260 seconds in the one-stage assays, 210 seconds in the FVIII chromogenic assay and 520 seconds in the FIX chromogenic assay. All assays were validated for clinical patient care. Siemens control N and control P were used for internal quality control. The methods were also evaluated by participation in the ECAT external quality program. In all quality controls, our coagulation laboratory has performed well (at or around the average) in the period covering the central study measurements. All measurements were performed on a CS-2500 analyzer and in bulk. The same citrated plasma sample was used for both assays. The final results obtained centrally were compared to the most recent baseline factor level that was measured at the local laboratory. In case of marked differences, the local center was contacted to double check whether the sample was drawn after a washout period.

Literature search

To identify all mutations associated with assay discrepancy, we performed a literature search. PubMed was searched using the key terms “hemophilia” and “assay discrepancy” on January 6, 2022.

Studies from peer-reviewed journals were included when information was provided on specific *F8* or *F9* mutations associated with assay discrepancy and the full text was available. Catchments areas were evaluated to ensure that similar patient cohorts in different studies were not included twice. The reference list of a recent literature review on assay discrepancy in hemophilia A[6] was checked to retrieve any additional studies. Data was collected by a single reviewer (A.Z.) on study setting, discrepancy definition and mutations resulting in assay discrepancy with direction (standard or reverse) including the total number of persons with this mutation in the total cohort if available.

Statistical analysis

Data are presented descriptively as medians with interquartile ranges (IQR) for continuous variables and as absolute numbers with proportions (%) for categorical variables. Scatterplots were constructed to visualize relationships between factor activity level as measured with both assays. In addition, Bland-Altman plots were created to visualize the agreement[14]. The first plot was presented as ratio (OSA over CA) against the mean of the two assay results. The second plot was presented as the absolute difference (OSA – CA) against the mean of the two assay results. No limits of agreement were calculated as ratio and difference did not follow a normal distribution. Instead, upper and lower bounds of the primary assay discrepancy definitions were shown in the plots. The analyses were performed using SPSS version 28 (IBM SPSS Statistics, Chicago, IL, USA).

Data sharing statement

For original data, please contact m.coppens@amsterdamumc.nl.

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Results

Population characteristics

A total of 304 persons participated in the DYNAMO study[15] and for 227 persons central measurements were performed. Seven persons were excluded as assay results showed differences in comparison to the expected values and were confirmed as not baseline or unknown after double checking with the local center. As a result, the present analysis included 220 persons, comprising 175 participants (80%) with hemophilia A and 45 participants (20%) with hemophilia B. The median lowest historical factor level measured at the local laboratories was 9 IU/dL (IQR 4-16). This resulted in 32% and 68% classified as moderate and mild hemophilia, respectively. Most participants received on demand treatment (94%). Table 1 summarizes the characteristics of the cohort.

Extent of assay discrepancy

In hemophilia A, the median FVIII activity level was 10.6 IU/dL (IQR 4.8-18.3) and 9.7 IU/dL (IQR 4.1-19.3) as measured with respectively OSA and CA. In hemophilia B, the median FIX activity level was 10.7 IU/dL (IQR 4.6-17.5) and 10.1 IU/dL (IQR 4.3-15.5) as measured with respectively OSA and CA. An assay discrepancy defined according to the ISTH definition (ratio of >2.00 or <0.50) was detected in three persons (1%) corresponding to 2 persons with hemophilia A and 1 person with hemophilia B. In six persons (3%) an absolute difference of >10 IU/dL between assay measurements was found including 4 persons with hemophilia A and 2 persons with hemophilia B. With the more lenient discrepancy definitions, respectively 8% and 5% of this cohort exhibited relative (ratios >1.50 or <0.67) and absolute (difference >5 IU/dL) discrepancies (Table 2). Detailed information on these persons are presented in Table 3. The correlation between assay results is demonstrated for the total cohort (Figure 1) and hemophilia A and B separately (Figure 2). The agreement between assay results is illustrated in Figure 3. The median difference between the assays was generally low with 1.1 IU/dL (IQR 0.5-2.1) for the total cohort, 1.1 IU/dL (IQR 0.5-2.0) in case of higher one-stage results and 1.3 IU/dL (IQR 0.7-2.4) in case of higher chromogenic results. Larger ratios were observed within the lower factor activity range and larger absolute differences with increasing factor levels.

Mutations with assay discrepancy

The *F8*/*F9* gene mutation was available in 152 of 220 (69%) of our study population. A total of 16 mutations were associated with assay discrepancy in our study according to any of the lenient definitions (Table 3). Five of these mutations were also identified in 12 other study participants (Arg717Trp n=4, Arg1960Gln n=2, Pro149 n=4, Arg550His n=1, Ser2030Asn n=1). None of them exhibited assay discrepancy (Table S1).

The literature search revealed 90 mutations that have been associated with assay discrepancy at least once (Table S2). In our cohort, 60 participants (27% of cohort) carried any of these mutations. When restricted to mutations previously reported in at least 3 persons (Table S3[7,8,10,11,16-27]) a total of 13 study participants were identified carrying any of these mutations as presented in Table 4 (Arg1985Gln n=2, Arg550Cys n=1, Arg550His n=2, Arg717Trp n=5, Leu1951Phe n=1, Thr314Ala n=1, Arg19His n=1). In only two of them an assay discrepancy was measured according to our strict definitions and in a third participant an assay discrepancy was measured according to the more lenient definition (absolute difference >5 IU/dL). In the other ten persons with mutations that were frequently reported to demonstrate assay discrepancy, this could not be reproduced in our study. Interestingly, in one participant with discrepancy carrying the Arg717Trp mutation, the CA results were >10 IU/dL higher in comparison to the OSA results. This is opposite to the direction of discrepancy typically found for this mutation as other persons with Arg717Trp in this study and in previous literature had higher one-stage results in comparison to chromogenic results. A similar contradictory finding was also observed in two participants that carried the Arg1985Gln mutation. This mutation was previously reported to result in standard assay discrepancy (OSA > CA) but demonstrated slightly higher CA results in our study, although this was not large enough to be classified as assay discrepancy.

Comparison with local measurements

In 9 participants from 5 different centers, assay discrepancy was previously identified with historical measurements at the local center. Strikingly, the results in all these persons exhibited no assay discrepancy with the central measurements (Table S4). In 4 of 5 persons with multiple measurements over time, the results were also inconsistently discrepant at these different time points.

Discussion

In this international cohort of 220 persons with non-severe hemophilia A and B, we found very little discrepancy between central one-stage and chromogenic assay measurements. Even with more lenient discrepancy definitions, the vast majority (90%) of this cohort had no discrepant results. This is in contrast to previous studies that reported assay discrepancy in around one-third of persons with non-severe hemophilia. In addition, the majority of persons with a *F8*/*F9* gene mutation previously associated with assay discrepancy exhibited no discrepant results in our study. This suggests that assay discrepancy seems to be largely determined by laboratory variables.

Prevalence of assay discrepancy

In non-severe hemophilia A, assay discrepancy has been frequently addressed by previous studies. A recent literature review including 18 articles reported an estimated pooled prevalence of 36% (95% CI 28-44) assay discrepancy (range 12-84% in the individual studies)[6]. In non-severe hemophilia B, data on assay discrepancy is scarce with only one article currently published[10]. This study examined 32 persons with non-severe hemophilia B and found assay discrepancy in 25% of the study population. In our study, a strikingly lower prevalence of significant assay discrepancy was found for both hemophilia A (2/175 persons – 1%) and hemophilia B (1/45 persons – 2%). Some differences in prevalence between studies may be related to the criteria used to define assay discrepancy. While we used the definition of the ISTH-SSC for our primary outcome, some other studies applied less stringent definitions. However, even when we applied these more lenient definitions (ratio >1.50 or <0.67 and absolute difference >5 IU/dL) we still found less than 10% discrepancy. The impact of baseline factor level on discrepancy is also reflected by our Bland-Altman plots showing larger ratios with lower factor levels and larger absolute differences with higher factor levels. As such, assay discrepancy based on ratio difference is more likely to be observed in persons with moderate hemophilia and discrepancy based on absolute difference more in those with higher factor activities. Furthermore, heterogeneity may arise from differences in design, study population and factors causing laboratory measurement variation.

Mutations and assay discrepancy

In previous studies, assay discrepancy has been linked to several specific mutations, supporting the genetic background of this phenomenon[7,11]. In our study, we did not confirm these observations. The mutations that resulted in assay discrepancy in our study, were also identified in 12 other study participants of whom none had discrepant results. Furthermore, 27% of our study cohort carried a mutation that was reported to display assay discrepancy in previous literature, but that could not be

replicated in our study. Even when we further restricted our analysis to frequently reported mutations associated with discrepancy (reported in at least 3 patients in previous literature) in only one participant a significant assay discrepancy was present. While the direction of assay results was as expected for most persons, we also observed inconsistencies. For the Arg717Trp mutation we found one participant with an assay discrepancy (difference >10 IU/dL) with higher CA results, while four other participants with this mutation had higher OSA results. Conflicting results have also been reported for the Arg546Trp mutation (not present in our study) resulting in both standard and reverse assay discrepancy among 3 different studies[7,11,21]. To the same extent, the Arg191His mutation caused assay discrepancy in 6/6 persons with hemophilia B in the study by Kihlberg et al[10]. In our study, we included one participant with this mutation in whom similar results by both assays were found. Our results may suggest that the impact of specific mutations varies depending on assay conditions, further supporting the notion that the issue of assay discrepancy is mainly driven by laboratory-related factors.

Measurement variation

Many different factors may contribute to variation in measured factor activity. First, pre-analytical variables can influence the assay measurements, including inappropriate blood collection, processing, storage or transport[28]. Second, analytical factors may affect measured clotting factor activity. Inter-assay and inter-laboratory variability are frequently observed with estimated variation coefficients around 5-20% and 15-25%, respectively[28]. Also in our own dataset we observed inter-laboratory variability as reflected by differences between central and local measurements. These findings are in line with previous work investigating the analytical variation in FVIII activity in the ECAT external quality assessment program[29]. In that study, OSA results varied between set-ups from several manufacturers and was partially explained by the calibrator value[29]. Other studies assessing different one-stage reagents reported varying factor activity results for FVIII[22] and FIX levels[30]. As a consequence, the presence of assay discrepancy and in some cases the direction may also depend on the reagents and set-up used. The incubation time in the chromogenic assay also has a potential impact as longer incubation times can result in lower measured factor activity levels in some patients[31]. Furthermore, many previously reported assay discrepancies have been based on one-stage and two-stage legacy assays. While chromogenic assays are considered two-stage assays, historically clot-based two-stage assays differ from the modern chromogenic assays. Chromogenic assays have only been available on a larger scale since the last decade and show a considerable variability in the duration of the incubation phase, which may affect different mutations differentially. In addition, the varying use of kinetic or end-point methodology in the chromogenic assay may add to any differences observed. It is likely that all such factors, including reagents, set-

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ups and duration of incubation largely determine the magnitude of assay discrepancy. In our study, it is plausible that the little discrepancy observed is influenced by the particular single one-stage and chromogenic assay used. Third, in general, intra-individual variation in baseline factor levels may contribute to measurement variability. Previous work demonstrated that intra-individual variation explained 45% of the variability in FVIII levels adjusted for age, mutation and hemophilia treatment center[32]. This raises the question to what extent laboratory issues or other determinants such as stress account for the observed variance. Hence, multiple variables can influence measured factor activity and may contribute to the marked heterogeneity in results that are reported in this area of work.

Strengths and limitations

The DYNAMO study included a relatively large cohort of persons with non-severe hemophilia within a multicenter international setting. All assays were performed centrally in bulk and using the same plasma sample to reduce potential analytical variability as much as possible. A strength was that we included patients with hemophilia B as only one previous smaller study has focused on assay discrepancies in this population[10]. The local laboratories were provided a protocol with specifications to ensure similar processing and storage of samples. However, remaining variation arising from other pre-analytical factors such as errors in blood collection or inadequate temperature control at local sites cannot be completely ruled out. Another strength was the presence of persons with mutations previously associated with assay discrepancy to put our observations in perspective. Limitations of the present work include that mutation data was missing for 31% of our cohort. Furthermore, it may be that a longer incubation time for the chromogenic assay could have resulted in more discrepancy, nonetheless demonstrating that laboratory factors have critical impact on measured activity. As we only used one type of OSA and CA, it remains unknown whether the use of alternative kits would have resulted in different findings. Unfortunately, our data collection did not include details on the methodology of local factor level measurements (i.e. reagent, manufacturer) which would allow a more direct comparison between central and local measurements. Lastly, it needs to be emphasized that the majority of this study cohort has been historically diagnosed with hemophilia based on results from a OSA as outlined below.

Study cohort

In the DYNAMO study, persons with non-severe hemophilia aged 12-55 years were eligible for inclusion. The median age at diagnosis was 3 years (IQR 0-12)[15] and consequently the majority of this population has been diagnosed based on OSA results. This may have led to a potential selection bias in which persons with normal OSA results but a factor activity of 35 IU/dL or lower with the CA have

not been included. As such, the study population may lack persons with (very) mild hemophilia that have not been accurately identified as hemophilia patient in the past. However, most historical hemophilia cohorts reporting assay discrepancy are prone to the same selection mechanism and therefore this does not explain why the assay discrepancy in our study is smaller than previously reported in other studies of persons diagnosed with hemophilia with a (OSA) factor activity of 2-35 IU/dL. Nevertheless, considering the potential selection bias, our findings do not obviate the need to perform both assays in the work-up of a bleeding patient.

Clinical implications

Accurate measurement of FVIII and FIX activity levels is vital to ensure adequate disease classification and management in hemophilia. Awareness is required that factor activity levels vary and depend on a range of laboratory- and patient-related variables. A low prevalence of assay discrepancy was observed in this cohort, which may be attributed to the particular measurement conditions used in our central measurements. This strengthens our hypothesis that assay discrepancy is largely driven by laboratory variation. Fortunately, in most patients the factor activity will lead to a correct diagnosis whether it is measured by the OSA or CA. However, there is a subgroup of 5-10% of patients with mild hemophilia A that may have normal results by either OSA or CA[6,34] and in whom diagnosis will be missed when the diagnostic work-up is restricted to one assay. Thus, we support recommendations to use both the OSA and CA in the diagnostic work-up of non-severe hemophilia[6,9,12,33]. For monitoring and routine care, one assay may be sufficiently informative for most clinically relevant decision-making when analytical variability is minimalized. When assay discrepancy does occur, uncertainties still exist on which assay is most reflective of the 'true' level, consistent with the clinical bleeding phenotype[34]. It has been suggested that the lowest factor level by any assay correlates best to the phenotype[5], while some studies suggest that CA better reflects bleeding tendency[20,34-36]. Interestingly, some previous studies reported on persons diagnosed with hemophilia based on low OSA results that were identified following investigation of a prolonged APTT, rather than based on a clinical suspicion of hemophilia[26,37]. This raises the question whether the low OSA values found in that study represented clinically relevant results. Within our study we had a good agreement between assay results which precludes any exploration on which assay best reflects the bleeding phenotype. Awaiting harmonization of factor level measurements, we suggest that laboratory results should be primarily evaluated in the context of the persons' clinical profile. Considering the important influence of laboratory conditions on the presence and magnitude of assay discrepancy, we suggest repeated testing in case of discrepancy. As this study illustrates, some persons labeled as discrepant may exhibit concordant activity levels under different circumstances.

Further standardization of FVIII and FIX measurements is required to improve adequate diagnosis, clinical management and future research into assay discrepancy in non-severe hemophilia A and B.

Conclusion

In conclusion, we found little assay discrepancy in this international cohort of 220 persons with non-severe hemophilia A and B, even in those persons with mutations previously associated with discrepancy. This suggests that laboratory-related factors contribute largely to the presence and magnitude of assay discrepancy.

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Authorship contributions

K. Fijnvandraat designed the study. A. Zwagemaker analysed the data and wrote the manuscript. A. Zwagemaker, F.R. Kloosterman, S.C.Gouw and M. Coppens interpreted the data and drafted the manuscript. S.C. Gouw, S. Boyce, P. Brons, M.H. Cnossen, P.W. Collins, J. Eikenboom, C. Hay, S. Jackson, M.J.H.A. Kruip, B. Laros-van Gorkom, C. Male, L. Nieuwenhuizen, S. Shapiro, K. Fijnvandraat and M. Coppens collected data or supervised data collection. R.C.C. Hengeveld and C.A.M. Klopper-Tol performed or supervised the assay measurements and interpreted the data from a laboratory perspective. All authors reviewed this work and approved the final version of the manuscript.

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Disclosures of conflicts of interest

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All members of the DYNAMO study group are listed in the supplemental material.

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Figure legends

Figure 1. Scatterplot of measured factor activity with one-stage vs. chromogenic assay for the total cohort. The colored dots represent persons with significant assay discrepancy (red dots), persons with an absolute difference >10 IU/dL between the assay results (yellow dots) and persons meeting both these criteria (orange dots). The line reflects x=y.

Figure 2. Scatterplots of measured factor activity with one-stage vs. chromogenic assay for hemophilia A and hemophilia B. The colored dots represent persons with significant assay discrepancy (red dots), persons with an absolute difference >10 IU/dL between the assay results (yellow dots) and persons meeting both these criteria (orange dots). The line reflects x=y.

Figure 3. Bland-Altman plots of measured factor activity with one-stage vs. chromogenic assay as (A) ratio against mean and (B) absolute difference against mean. The colored dots represent persons with significant assay discrepancy (red dots), persons with an absolute difference >10 IU/dL between the assay results (yellow dots) and persons meeting both these criteria (orange dots). (A) The bold line represents the mean ratio of 1.1 of one-stage over chromogenic results. The dashed lines represent the upper and lower ratios to define significant assay discrepancy (ratio >2.00 or <0.50). (B) The bold line represents the mean absolute difference of 0.1 of one-stage minus chromogenic results. The dashed lines represent the upper and lower differences to define an absolute difference >10 IU/dL.

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Table 1. Patient characteristics of the study cohort.

	Total cohort *n = 220*	**Hemophilia A** *n = 175*	**Hemophilia B** *n = 45*
Age [years]	39 (26-49)	40 (26-49)	36 (27-47)
Hemophilia severity			
moderate	71 (32)	54 (31)	17 (38)
mild	149 (68)	121 (69)	28 (62)
Lifetime lowest factor activity [IU/dL]	9 (4-16)	9 (5-16)	9 (3-15)
Treatment regimen			
full prophylaxis	8 (4)	6 (3)	2 (4)
intermittent prophylaxis	5 (2)	4 (2)	1 (2)
on demand	207 (94)	165 (94)	42 (93)

Characteristics are presented as medians with corresponding interquartile range (IQR) or as absolute numbers with proportions (%).

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Table 2. Frequencies of assay discrepancy including primary and secondary outcomes.

	Total cohort *n = 220*	**Hemophilia A** *n = 175*	**Hemophilia B** *n = 45*
Primary outcomes			
Ratio >2.00 or <0.50	3 (1)	2 (1)	1 (2)
Absolute difference >10 IU/dL	6 (3)	4 (2)	2 (4)
Secondary outcomes			
Ratio >1.50 or <0.67	17 (8)	13 (7)	4 (9)
Absolute difference >5 IU/dL	11 (5)	8 (5)	3 (7)

Presented as absolute numbers with proportions (%).

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Table 3. Characteristics of persons with assay discrepancy. Results meeting the primary discrepancy definitions are highlighted in dark orange. Results meeting the more lenient discrepancy definitions are highlighted in light orange.

ID	Hemophilia type	Age (years)	OSA (IU/dL)	CA (IU/dL)	Ratio OSA/CA	Absolute difference (IU/dL)	Mutation protein change
1	A	23	20.0	6.0	3.33	14.0	-
2	A	28	7.8	3.5	2.23	4.3	Arg550Cys*
3	B	37	23.5	10.1	2.33	13.4	Thr84Ile
4	A	43	29.1	40.3	0.72	11.2	Arg717Trp*
5	A	51	22.8	37.1	0.61	14.3	-
6	A	43	29.4	49.2	0.60	19.8	Phe698Ser
7	B	51	44.3	23.1	1.92	21.2	-
8	A	53	0.8	0.4	2.00	0.4	-
9	A	45	6.3	3.2	1.97	3.1	Arg1960Gln
10	A	31	6.0	3.1	1.94	2.9	Gly498Arg
11	A	25	4.8	2.5	1.92	2.3	-
12	A	39	1.3	0.7	1.86	0.6	Arg2182His
13	A	14	3.5	2.1	1.67	1.4	-
14	A	29	4.3	2.7	1.59	1.6	Pro149Arg
15	A	54	5.3	8.9	0.60	3.6	Asp588Glu
16	A	44	1.7	3.2	0.53	1.5	Arg391His
17	B	40	7.8	4.6	1.70	3.2	Arg379Pro
18	B	52	2.2	1.3	1.69	0.9	Arg379Gln
19	A	21	28.7	22.2	1.29	6.5	Arg550His*
20	A	31	29.3	23.0	1.27	6.3	Ile1901Thr
21	A	14	31.8	37.2	0.85	5.4	Ser2030Asn
22	A	22	17.0	22.3	0.76	5.3	Arg2169Cys
23	B	56	23.5	16.1	1.46	7.4	-

* Mutation previously reported to be associated with assay discrepancy in at least 3 persons.

CA: chromogenic assay, OSA: one-stage assay.

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Table 4. Mutations described in literature associated with assay discrepancy and results in our cohort. Results meeting the primary discrepancy definitions are highlighted in dark orange. Results meeting the more lenient discrepancy definitions are highlighted in light orange.

Literature		Central study measurements					
Mutation protein change	**Direction of assay results***	**ID**	**OSA (IU/dL)**	**CA (IU/dL)**	**Ratio OSA/CA**	**Absolute difference (IU/dL)**	**Direction of assay results***
Hemophilia A							
Thr314Ala	Reverse	1	17.7	22.0	0.80	4.3	Reverse
Arg550Cys	Standard	2	7.8	3.5	2.23	4.3	Standard
Arg550His	Standard	3	28.7	22.2	1.29	6.5	Standard
		4	31.0	27.7	1.12	3.3	Standard
Arg717Trp	Standard	5	29.1	40.3	0.72	11.2	Reverse
		6	20.5	18.7	1.10	1.8	Standard
		7	30.4	29.2	1.04	1.2	Standard
		8	29.7	28.7	1.03	1.0	Standard
		9	23.2	23.5	0.99	0.3	Standard
Leu1951Phe	Standard	10	6.9	5.4	1.28	1.5	Standard
Arg1985Gln	Standard	11	21.5	22.6	0.95	1.1	Reverse
		12	27.5	30.7	0.90	3.2	Reverse
Hemophilia B							
Arg191His	Reverse	13	6.6	7.1	0.93	0.5	Reverse

CA: chromogenic assay, OSA: one-stage assay.

***** Standard defined as higher one-stage compared to chromogenic results. Reverse defined as higher chromogenic compared to one-stage res

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Assay results in non-severe hemophilia

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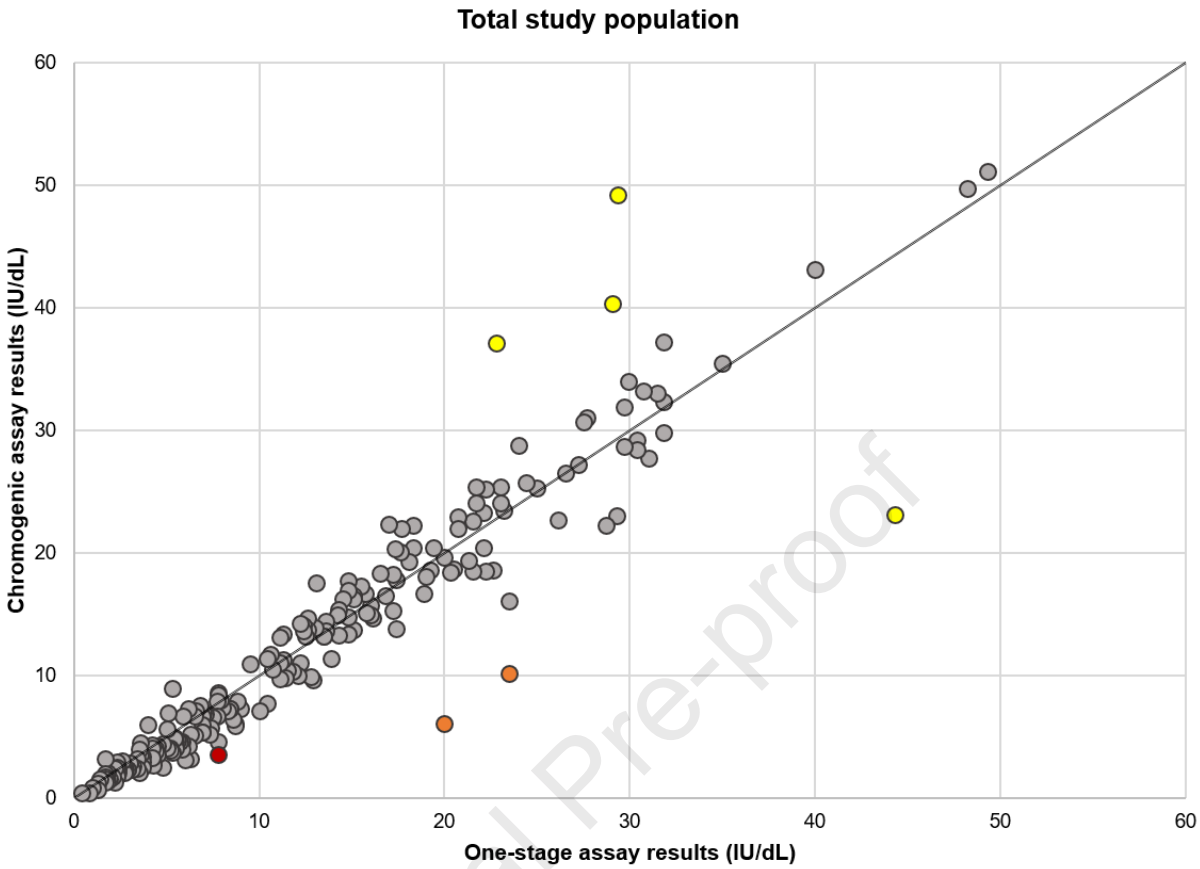

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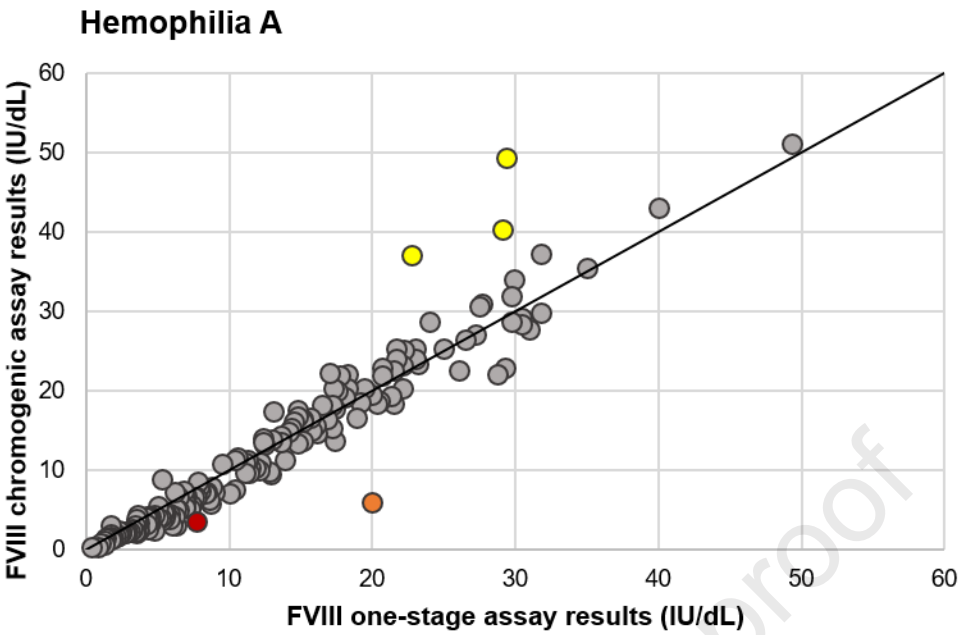

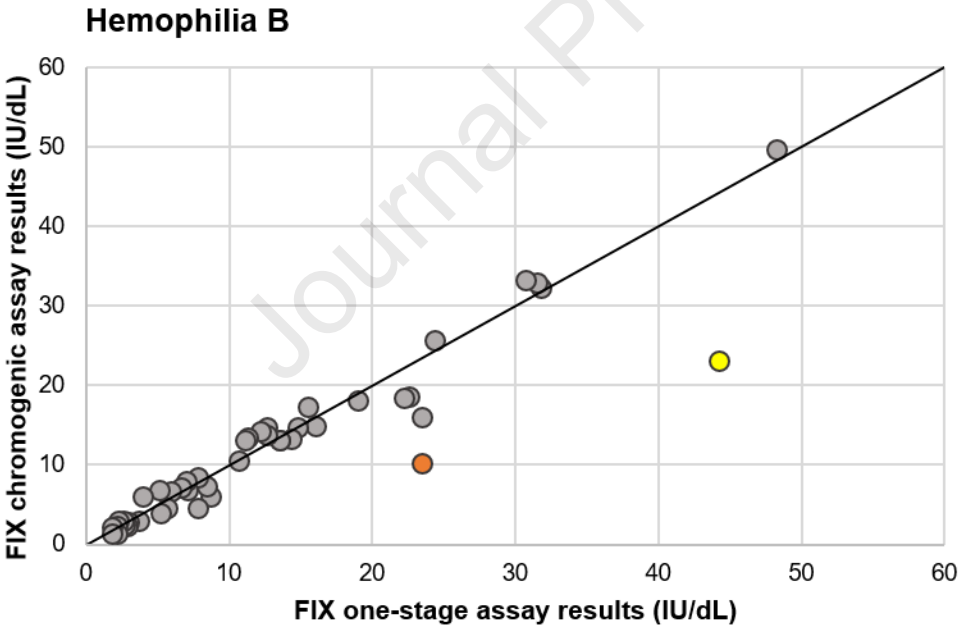

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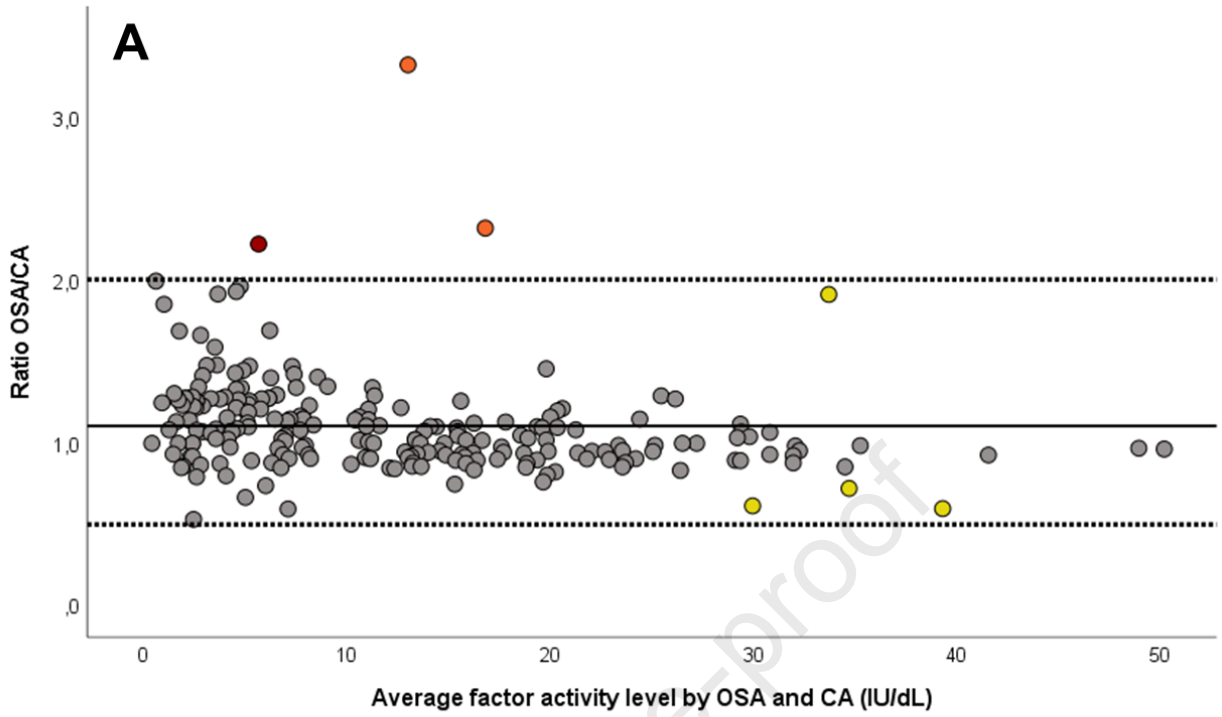

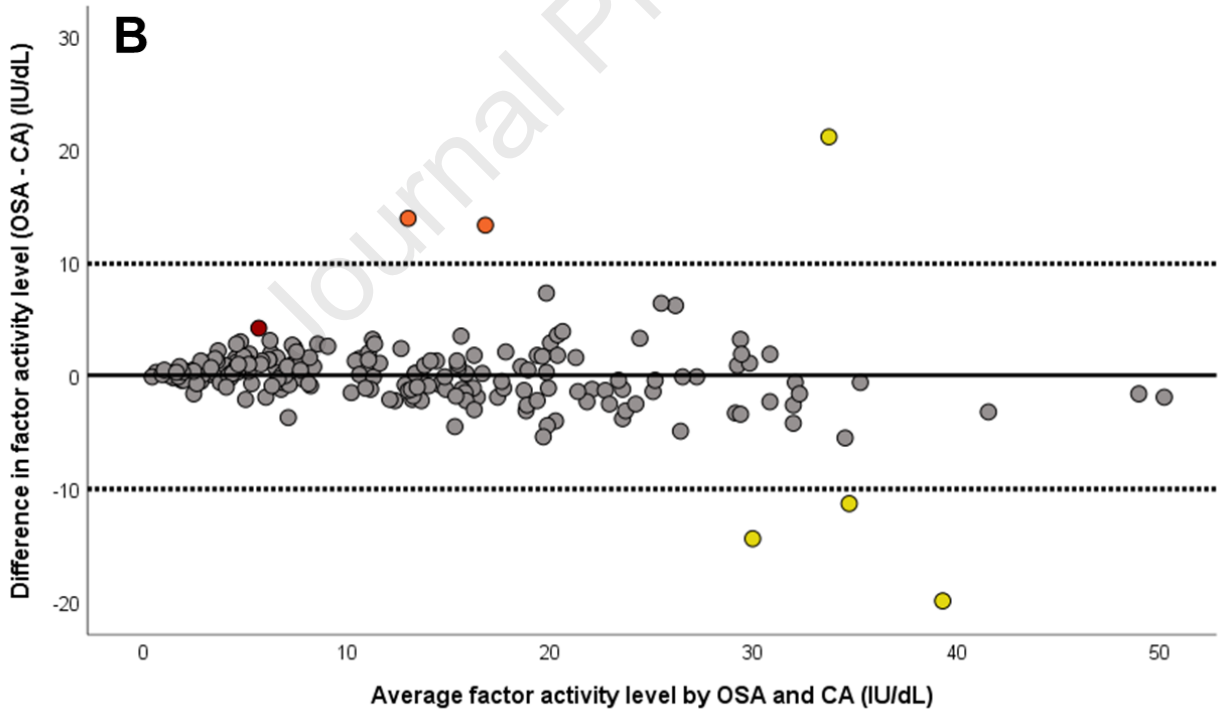

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STROBE Statement—checklist of items that should be included in reports of observational studies

	Item No.	Recommendation	Page No.	Relevant text from manuscript
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	2	"Large international cohort"
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	2	In abstract
Introduction				
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	3	In introduction
Objectives	3	State specific objectives, including any prespecified hypotheses	4	Therefore, the aim of this study was to investigate the extent of assay discrepancy in persons with non-severe hemophilia A and B in a multicenter international setting. Additionally, assay discrepancy was assessed in a subgroup of persons with mutations associated with discrepancy in previous literature.
Methods				
Study design	4	Present key elements of study design early in the paper	5	In methods
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	5	In methods
Participants	6	(*a*) *Cohort study*—Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up *Case-control study*—Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls *Cross-sectional study*—Give the eligibility criteria, and the sources and methods of selection of participants	5	In methods
		(*b*) *Cohort study*—For matched studies, give matching criteria and number of exposed and unexposed *Case-control study*—For matched studies, give matching criteria and the number of controls per case	N/A	N/A

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Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	5	In methods
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	5-6	In methods
Bias	9	Describe any efforts to address potential sources of bias	11-12	In discussion
Study size	10	Explain how the study size was arrived at	8	In results
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	6-7	In methods
Statistical methods	12	(a) Describe all statistical methods, including those used to control for confounding	6-7	In methods
		(b) Describe any methods used to examine subgroups and interactions	5	In methods
		(c) Explain how missing data were addressed	8 12	Missing data for assay analyses % is stated in results and missing data for mutation data stated in discussion
		(d) Cohort study—If applicable, explain how loss to follow-up was addressed Case-control study—If applicable, explain how matching of cases and controls was addressed Cross-sectional study—If applicable, describe analytical methods taking account of sampling strategy	N/A	N/A
		(e) Describe any sensitivity analyses	5	In methods – assay discrepancy was calculated according to different discrepancy definitions
Results				
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	8	In results
		(b) Give reasons for non-participation at each stage	8	In results (central lab analyses were not available for the complete cohort)
		(c) Consider use of a flow diagram	N/A	N/A
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	8 18	In results and table 1

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		(b) Indicate number of participants with missing data for each variable of interest	8	In results
		(c) *Cohort study*—Summarise follow-up time (eg, average and total amount)	N/A	N/A
Outcome data	15*	*Cohort study*—Report numbers of outcome events or summary measures over time	8 19	In results and table 2
		Case-control study—Report numbers in each exposure category, or summary measures of exposure	N/A	N/A
		Cross-sectional study—Report numbers of outcome events or summary measures	N/A	N/A
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	8	In results
		(*b*) Report category boundaries when continuous variables were categorized	N/A	N/A
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	N/A	N/A
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	8-9 19-21	In results and tables 2-4
Discussion				
Key results	18	Summarise key results with reference to study objectives	10	In discussion
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	12	In discussion
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	10-12	In discussion
Generalisability	21	Discuss the generalisability (external validity) of the study results	12	In discussion
Other information				
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	14	In acknowledgements