BMJ Open

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Journal:	*BMJ Open*
Manuscript ID	bmjopen-2016-015636
Article Type:	Protocol
Date Submitted by the Author:	20-Dec-2016
Complete List of Authors:	Heida, Anke; Univ Groningen, Pediatric Gastroenterology Vijver, Els; Universitair Ziekenhuis Antwerpen, Pediatric gastroenterology Muller Kobold, Anneke; Univ Groningen, Laboratory Medicine Rheenen, Patrick; Univ Groningen, Pediatric Gastroenterology
Primary Subject Heading:	Gastroenterology and hepatology
Secondary Subject Heading:	Paediatrics
Keywords:	S100A12 protein, S100 proteins, diagnostic accuracy, Inflammatory bowel disease < GASTROENTEROLOGY, Screening

Selecting children with suspected inflammatory bowel disease for endoscopy with the calgranulin C or calprotectin stool test: protocol of the CACATU study.

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Keywords: S100A12 protein; S100 proteins, Inflammatory bowel disease, Screening

Trial registration: ClinicalTrials.gov identifier: NCT02197780 (registered 21 July 2014, last updated 27 August 2016)

Version: 1

ABSTRACT:

INTRODUCTION: The introduction of the fecal calprotectin (FC) test to screen children with chronic gastrointestinal complaints has helped the clinician to decide whether or not to subject the patient to endoscopy. In spite of this, a considerable number of patients without IBD is still scoped. Fecal calgranulin C (S100A12) is a marker of intestinal inflammation that is potentially more specific for inflammatory bowel disease (IBD) than FC, as it is exclusively released by activated granulocytes.

OBJECTIVE: To determine whether the specificity of S100A12 is superior to the specificity of FC without sacrificing sensitivity in patients with suspected IBD.

METHODS: An international prospective cohort of children with suspected IBD will be screened with the existing FC stool test and the new S100A12 stool test. The reference standard (endoscopy with biopsies) will be applied to patients at high risk of IBD, while a secondary reference (clinical follow up) will be applied to those at low risk of IBD. The differences in specificity and sensitivity between the two markers will be calculated.

ETHICS: This study is submitted to and approved by the Medical Ethics Review Committee (MEC) of the University Medical Center Groningen (the Netherlands) and the Antwerp University Hospital (Belgium).

Trial registration: ClinicalTrials.gov identifier: NCT02197780 (registered 21 July 2014)

STRENGTHS AND LIMITATIONS OF THIS STUDY

- First head-to-head comparison of calprotectin and S100A12 to select children with chronic gastrointestinal complaints for endoscopy
- Unbiased selection of patients from secondary and tertiary care settings in whom it is clinically sensible to suspect inflammatory bowel disease
- Different reference standards for patients with high and low risk of IBD; may invoke good treatment-free prognosis as proof of non-IBD
- Bayesian correction method to adjust for differential-verification bias

INTRODUCTION

Background and rationale

The introduction of the calprotectin stool test to screen children with chronic gastrointestinal complaints has helped the clinician to decide whether or not to refer the patient for endoscopy.[1–4] Children with normal screening test results (≤ 50 µg/g) have a low probability of inflammatory bowel disease and should not be exposed to the invasive reference test.[5] Children with elevated calprotectin levels have a high probability of IBD and require referral to an endoscopy unit for endoscopic evaluation of upper and lower gastrointestinal tract.[1,4,5] Although use of the calprotectin stool test rarely misses a child with IBD, the number of false positive cases who are scoped is considerable.[1,5] Fecal calgranulin C (S100A12) is a marker of intestinal inflammation that is potentially more specific for inflammatory bowel disease (IBD) compared to fecal calprotectin, as it is exclusively released by activated granulocytes.[6]

Objectives

We hypothesize that a referral strategy based on fecal S100A12 will reduce the number of children wrongly selected for endoscopy as compared to a calprotectin-based strategy. The primary objective is to determine whether the specificity of S100A12 is superior to the specificity of calprotectin without sacrificing sensitivity. The secondary objective is to calculate the diagnostic accuracy characteristics and best cut-offs for both S100A12 and calprotectin.

METHODS

Design

The CACATU study is a prospective, observational, multicenter, diagnostic accuracy study with a paired design. A cohort of children with suspected IBD is screened with the calprotectin stool test (existing test) and with the S100A12 stool test (new test). Confirmation of the target condition (IBD) is based on endoscopy with biopsies (reference standard) or clinical follow up (secondary reference standard).

Study setting

Study participants will be recruited from fifteen general teaching hospitals and one academic center in the Netherlands, and from one general hospital and two academic centers in Flanders, Belgium. The names of all participating centers can be found in the trial registry (www.clinicaltrials.gov). The principal investigators at the various sites are general pediatricians or pediatric gastroenterologists. Six participating centers (3 academic and 3 general hospitals) have a pediatric endoscopy unit.

Eligibility criteria

Patients were eligible if they were between 6 and 18 years old and presented with at least one major criterion or two minor criteria suggestive of IBD (Table 1).

Outcomes

The primary outcome is the difference in specificity between FC and S100A12. Secondary endpoints are the difference in sensitivity and the diagnostic accuracy characteristics (sensitivity, specificity, positive predictive value, negative predictive value, area under the curve, best cut-off point) for both markers individually. All diagnostic accuracy characteristics will be calculated with predefined cut-off points that have been documented in the medical literature, and with best cut-off points based on receiver operator characteristic (ROC) curves.

Intervention

Patients who fulfill the inclusion criteria will be managed according to a calprotectin-based referral strategy. The treating clinician will determine the risk stratification (high vs. low risk of IBD), based on the combination of FC result, presenting symptoms and blood tests. In general we expect that those participants with calprotectin levels over 50 µg/g without colon pathogens are likely to be referred to endoscopy (the preferred reference standard). Patients with normal calprotectin levels are likely to have a low probability of IBD and receive clinical follow up (the alternative reference standard).

Participant study flow

Eligible participants will be invited for participation by their treating pediatrician. Baseline characteristics, date of birth, major and minor criteria (Table 1), use of NSAIDs and blood tests (Hemoglobin, C-reactive Protein, Erythocyte Sedimentation Rate, Alanine transaminase, and Gamma-Glutamyltranspherase) will be entered on a study website (Figure 2, step 1). Participants will be asked to defecate onto a stool collection sheet held above the toilet water and collect one sample with a screw top container with spoon (step 2). The stool sample is send to the UMCG hospital laboratory by ordinary mail. Immediately after arrival the level of FC will be measured. The residue will be split with one half stored at -80 degrees Celsius for S100A12 batch testing at a later stage, and the other half is used to determine enteric pathogens with a Polymerase Chain Reaction (PCR) technique (step 3). The PCR analysis will include Shiga-toxin producing E. Coli, E. Coli O157gen, Cryptosporidium, Dientamoeba Fragilis, Entamoeba histolytica, Giardia Lamblia, Salmonella, Shigella/EIEC and Campylobacter. Results of calprotectin test and PCR analysis will be uploaded on the website, and will then be made visible to the local clinician (step 4 & 5). The treating physician will receive an e-mail notification with an automated advice on the next best move (step 6). The choice of the reference standard (endoscopy or clinical follow up) is up to the clinician's discretion (step 7).

Timeline

The process from feces collection to completion of the non-invasive diagnostic work-up is supposed to last no longer than two weeks. We will exclude samples with a transport time that exceeds 7 days and we will perform a sub-analysis with those samples that are received within 4 days. In case of low risk of relapse the treating pediatricians will receive a reminder for clinical follow up 6 months after inclusion. The total running time of the study is 30

months, including 6 months to complete the follow up.

Sample size

The primary outcome of interest is the difference in specificity between the new test (S100A12) and the established test (FC). If the specificity of S100A12 is superior to the specificity of FC without sacrificing sensitivity, we can replace the old test by the new test. McNemar's test for paired data will be applied to compare specificities between both tests using a 2×2 table exclusively among non-IBD patients (Table 2). Study participants with concordant results ((+,+) or (- ,-)) do not distinguish between the two tests. The only information for comparing the sensitivities and specificities comes from those patients with discordant results ((+,-) or (- ,+)). Sample size calculation is based on recommendations in Hayen et al.[7] Weighed means of specificity of calprotectin were based on a recently published individual patient data meta-analysis.[4] We assumed that fecal S100A12 would lead to a 50% relative improvement of specificity (from 70% to 85%). The prevalence of IBD and non-IBD in the CACATU study cohort is expected to be similar to the prevalence that we found earlier[1], as the study participants will come from the same region and comparable eligibility criteria will apply. The sample size calculation was done with Power Analysis and Sample Size (PASS) software (version 11 for Windows). A sample size of 130 subjects with non-IBD achieves 80% power to detect a difference of 0.15 between the two diagnostic tests whose specificities are 0.70 and 0.85. This procedure used a two-sided McNemar test with a significance level of 0.05. The prevalence of non-IBD in the population is 0.64, and the proportion of discordant pairs is 0.23. We aim to include at least 250 participants, in order to correct for participants diagnosed with IBD (estimated 36%) and participants that will be lost to follow up (estimated 25%).

Recruitment

We asked all participating centers to predict how many eligible patients they could recruit during the enrolment period. Their estimates were based on the list of diagnoses of the previous year, and their estimated totals convinced us that reaching the target sample size is realistic. Retention will be promoted by sending automated reminders to the treating physician to complete the blanks in blood tests, and to re-assess patients with initial low probability of IBD after 6 months.

Test methods

Index tests

Fecal Calprotectin (FC)

FC levels will be measured with the fCal® ELISA test of BÜHLMANN Laboratories AG (Schönenbuch, Switzerland) according to manufacturer's instructions. A level of 50 µg/g is the predefined cut-off value.[2,4,5,8]

Fecal Calgranulin C (S100A12)

S100A12 levels will be measured by one experienced lab technician. The maximal duration of storage of the stool sample in our -80°Celsius freezer is 6 months. Analyses will be performed with a sandwich ELISA, trademark Inflamark® (CisBio Bioassays Codolet, France) on a Dynex DS2 Automated ELISA System (Alpha Labs, Easleigh, UK), according to the manufacturer instructions. In summary, after extraction step, 100 µL of pre-diluted samples will be transferred in duplicate into the corresponding wells coated with anti-S100A12 monoclonal antibody. Incubation time is 30 minutes, followed by three washing cycles with Tween 20. The next step is adding 100 µL of the second monoclonal antibody, anti S100A12 coupled to Horse Radisch-Peroxidase (HRP) followed by a second incubation period of 30 minutes and three washing cycles. Next, 100 µL of the substrate, tetramethyl benzidine, is pipetted in all wells. The wells are protected from light and after 10 minutes, the sulphuric acid stop solution is added. The absorbance will be read at 450 nm. For each duplicate, the mean optical density will be calculated and a calibration curve will be constructed. The curve will be plotted as a cubic regression with DS-matrix software, version 1.23 (Dynex technologies, Chantilly, USA). Purified human S100A12 will be used as calibrator (included in the kit).

The predefined cut-off value of S100A12 is 0.75 mg/kg, which is based on a reference value study among 120 healthy school-aged children and adolescents (pre-liminary, unpublished data).[9]

Reference tests

Endoscopy

Endoscopy will be the reference standard for patients at high risk of IBD. This procedure will be performed under anesthesia by an experienced pediatric gastroenterologist. Ideally, both upper and lower gastrointestinal tract will be evaluated according to the revised Porto criteria[10], and biopsies will be taken from every bowel segment. Histo-pathological examination will be performed by experienced histopathologists. Endoscopists and

histopathologists will have access to clinical information and FC test results, but will be blinded to the results of the S100A12 test.

Clinical follow up

This secondary reference will be applied to patients at low risk of IBD. Six months after study inclusion, the treating physician will receive a notification to enter a second evaluation of major and minor criteria (table 1). Blood tests will only be repeated when deemed necessary by the treating physician. In addition a second feces sample will be collected and send in for analysis. Patients who remain suspected of having IBD will be referred for endoscopy in second instance.

Rationale for choosing reference standard

Diagnostic endoscopic evaluation of the upper and lower gastrointestinal tract (including intubation of the terminal ileum) in combination with biopsies is the recommended test to diagnose IBD.[10] In children at high risk of IBD with negative endoscopy, small bowel imaging is encouraged.[10] All of these procedures are invasive and require bowel preparation. Endoscopy is mostly performed with the patient under general anesthesia. Although complications are rare, endoscopy is a burdensome procedure for a child. We found it unethical to expose children at low risk of IBD to endoscopy. Therefore, we decided to perform a secondary reference standard (clinical follow up) in patients at low risk for IBD and to adjust for its imperfection.[11]

Blinding

Laboratory personnel will be blinded to the patient's history, and to results of endoscopy and biopsy. Although calprotectin testing is done within 24 hours after arrival of the feces specimen and the residue is stored at -80 degrees Celsius for S100A12 batch testing at a later stage, sample labeling could theoretically link both fecal tests to one patient. Endoscopists and histopathologists will have access to clinical information and calprotectin test results, but will be blinded to the results of the S100A12 test.

Confidentiality and data management

Consecutive patients participating in the study will receive a unique study number. All demographic and medical data will be entered electronically on the study website by the local investigator and stored linked to this study number. Study investigators will receive access to

a secured study website. Local investigators are able to consult only data from participants from their own center. Feces samples will be marked with a study number label and sent to the Department of Laboratory Medicine at the UMCG. Results of calprotectin test and PCR for enteric pathogens will be uploaded on the website by the coordinating investigator and will be visible to the local clinician. At the end of the study the data entered on the study website will be cross-checked with the information in the local Electronic Health Databases. Data will be stored during the study period and until 15 years thereafter. When patients and their parents give permission, residual feces will be stored for a maximum period of 15 years for future diagnostic research. The researchers AH, EvdV and PvR will have access to the final trial dataset.

Statistical methods

Data analysis will be done with SPSS version 22.0 for Windows (SPSS, Chicago, IL, USA). Diagnostic accuracy characteristics (sensitivity, specificity, positive predictive value, negative predictive value) will be presented for both markers individually. McNemar's test for paired data will be applied to compare specificities between both tests using a 2×2 table exclusively among non-IBD patients. Likewise, sensitivities will be compared among IBD cases. We will primarily use pre-specified cut-off points of FC and S100A12. In second instance we will use the best cut-off points based on the receiver operating characteristic (ROC) curves for both FC and S100A12.

We will use a Bayesian correction method to adjust for differential-verification bias in the two reference standards in relation to latent IBD.[12] Based on clinical experience we defined a prior distribution. We assume that our reference standard endoscopy has 95 to 100% sensitivity, and 95 to 100% specificity to diagnose IBD, and that our secondary reference standard clinical follow up will have a sensitivity of 80 to 100%, and a specificity of 60 to 80%. Bayes factors will be calculated using JAGS ('Just Another Gibbs Sampler'), a free program licensed under GNU General Public License.

Finally, we will present the number of true- and false positive and true- and false negative results for four different scenarios: (1) FC screening only; (2) S100A12 screening only; (3) combination of FC and S100A12 in all patients; or (4) combination in a sub-set of patients with inconclusive FC results.

Missing values

We expect to encounter several types of missing outcomes in the CACATU study (Figure 1). In case the index test results are missing, the patient will be excluded from further analysis. In case of missing reference standard results (endoscopy refused, or follow up visit ignored) we will use multiple imputation matching for presenting symptoms and results of fecal markers.

Ethical approval

The study will be conducted according to the principles of the Declaration of Helsinki (59th version, October 2008). The Medical Ethics Review Committee of the University Medical Center Groningen is of the opinion that this study does not require approval according to the Dutch Medical Research Involving Human Subject Act (WMO). The Medical Ethics Review Committee of the Antwerp University Hospital approved the protocol. The legal guardian(s) from all participants, as well as children aged 12 and above, will need to give informed consent for participation and for storage of material for future research.
In case of important protocol amendments, both the Medical Ethics Review Committee and trial registry will be informed.

Dissemination policy

Authorship will be allocated to the authors of this protocol that initiated the study, completed with local investigators that will critically revise the content of the manuscript and include at least 5% of the total amount of participants. The local investigators that do not fulfill criteria for co-authorship will be mentioned in the acknowledgements.

Study status

The first trial participant was included in September 2014. It is anticipated that the trial will end in March 2017.

DISCUSSION

We aim to further improve the accuracy to distinguish patients with a high-risk of IBD from those with a low-risk of IBD with the ultimate goal to reduce the number of futile endoscopies. We will compare the established fecal marker FC with the relatively unknown fecal marker S100A12. The FC test has excellent sensitivity for IBD (0.92 to 0.98),[2–4,13] but its specificity, with point estimates varying between 0.60 and 0.68 [2–4,13] leaves a considerable proportion of non-IBD patients being exposed to an invasive procedure. Studies with fecal S100A12 showed diagnostic promise under ideal testing conditions in pre-selected groups of healthy children and children with IBD.[14–16] We only know of one report that compared FC and S100A12 in children presenting with gastrointestinal complaints.[15] The sensitivity and specificity of S100A12 for detection of IBD were both 97%, where FC had a sensitivity of 100% and specificity of 67%.[15]

Methodological biases

In this diagnostic accuracy study the performance of both stool tests will be assessed by verifying the results against endoscopy. Due to the invasive nature of this diagnostic procedure verification can be performed only in a subset of patients with a high risk of IBD. An alternative reference test (i.e. clinical follow up) will be given to the remainder of the patients. The drawback of this so-called differential-verification design is that the second reference test is of lesser quality. Simply adding the results of these two types of reference tests will lead to biased estimates of the overall test accuracy.[11] We plan to correct for this differential-verification bias by using a Bayesian approach, as described by De Groot et al.[12] Secondly, this study is a real life study, in which the decision to expose a child to endoscopy is based on the combination of presenting symptoms, physical examination and results of blood and stool tests, as is currently recommended by Dutch and international scientific societies. Blinding treating physicians for the FC results was therefore irrational and impractical. Knowledge of the FC level will influence the physicians' decision to refer a patient for endoscopic evaluation, which gives rise to a work-up bias.[17] Furthermore, endoscopists will not be blinded for the level of FC and therefore this may theoretically affect the endoscopists' assessment of the endoscopy (diagnostic review bias).

Implications for practice

If S100A12 has a better specificity than FC without sacrificing sensitivity, than S100A12 will be the dominant test to select patients for endoscopy. Replacing FC by S100A12 may then reduce the number of non-IBD patients being subjected to endoscopy. This will be good news for patients (less invasive tests), clinicians (shorter waiting lists for endoscopy), and

health insurance companies (reduction of healthcare cost).

Contributorship statement: PvR conceived the study. AH, EvdV, AMK and PvR initiated the study design. PvR is the grant holder. All authors contributed to refinement of the study protocol. All authors read and approved the final manuscript.
Cisbio Bioassays did not have a role in the design of this study and will not have any role during its execution, analyses, interpretation of the data, or decision to submit results.

Competing interests: This trial is supported by CisBio Bioassays, producer of the Inflamark® ELISA kit. PvR and AH received financial support from BÜHLMANN Laboratories AG (Schönenbuch, Switzerland) for other ongoing trials.

Funding: This study is funded by CisBio Bioassays (Codolet, France), developer and producer of the Inflamark® ELISA kit used to measure S100A12 in feces.

Data sharing statement: Not applicable for this studyprotocol, since we do not present any original data.

REFERENCES

1. Van de Vijver E., Schreuder AB., Cnossen WR., Muller Kobold AC., van Rheenen PF. Safely ruling out inflammatory bowel disease in children and teenagers without referral for endoscopy. *Arch Dis Child* 2012;**97**(12):1014–8.

2. Holtman GA., Lisman-van Leeuwen Y., Reitsma JB., Berger MY. Noninvasive Tests for Inflammatory Bowel Disease: A Meta-analysis. *Pediatrics* 2016;**137**(1):2015–126.

3. Henderson P., Anderson NH., Wilson DC. The diagnostic accuracy of fecal calprotectin during the investigation of suspected pediatric inflammatory bowel disease: a systematic review and meta-analysis. *Am J Gastroenterol* 2014;**109**(5):637–45.

4. Degraeuwe PLJ., Beld MPA., Ashorn M., Canani RB., Day AS., Diamanti A., et al. Faecal Calprotectin in Suspected Paediatric Inflammatory Bowel Disease: An Individual Patient Data Meta-Analysis. *J Pediatr Gastroenterol Nutr* 2015;**60**:339–46.

5. Heida A., Holtman GA., Lisman-Van Leeuwen Y., Berger MY., Van Rheenen PF. Avoid Endoscopy in Children With Suspected Inflammatory Bowel Disease Who Have Normal Calprotectin Levels. *J Pediatr Gastroenterol Nutr* 2016;**62**:47–9.

6. Foell D., Wittkowski H., Roth J. Monitoring disease activity by stool analyses: from occult blood to molecular markers of intestinal inflammation and damage. *Gut* 2009;**58**(6):859–68.

7. Hayen A., Macaskill P., Irwig L., Bossuyt P. Appropriate statistical methods are required to assess diagnostic tests for replacement, add-on, and triage. *J Clin Epidemiol* 2010;**63**(8):883–91.

8. Kostakis ID., Cholidou KG., Vaiopoulos AG., Vlachos IS., Perrea D., Vaos G. Fecal calprotectin in pediatric inflammatory bowel disease: a systematic review. *Dig Dis Sci* 2013;**58**(2):309–19.

9. Heida A., Kobold A., Wagenmakers L., Van De Belt K., Van Rheenen P. Reference values of fecal calgranulin C (S100A12) in school aged children and teenagers. *Journal of Pediatric Gastroenterology and Nutrition*, vol. 62. 2016. p. Suppl 1 (17).

10. Levine A., Koletzko S., Turner D., Escher JC., Cucchiara S., de Ridder L., et al. ESPGHAN revised porto criteria for the diagnosis of inflammatory bowel disease in children and adolescents. *J Pediatr Gastroenterol Nutr* 2014;**58**(6):795–806.

11. Naaktgeboren CA., de Groot JAH., Rutjes AWS., Bossuyt PMM., Reitsma JB., Moons KGM. Anticipating missing reference standard data when planning diagnostic accuracy studies. *BMJ* 2016;**352**:i402.

12. de Groot JAH., Dendukuri N., Janssen KJM., Reitsma JB., Bossuyt PMM., Moons KGM. Adjusting for differential-verification bias in diagnostic-accuracy studies: a Bayesian approach. *Epidemiology* 2011;**22**(2):234–41.

13. Lin J-F., Chen J-M., Zuo J-H., Yu A., Xiao Z-J., Deng F-H., et al. Meta-analysis: fecal calprotectin for assessment of inflammatory bowel disease activity. *Inflamm Bowel Dis* 2014;**20**(8):1407–15.

14. de Jong NSH., Leach ST., Day AS. Fecal S100A12: a novel noninvasive marker in children with Crohn's disease. *Inflamm Bowel Dis* 2006;**12**(7):566–72.

15. Sidler MA., Leach ST., Day AS. Fecal S100A12 and fecal calprotectin as noninvasive markers for inflammatory bowel disease in children. *Inflamm Bowel Dis* 2008;**14**(3):359–66.

16. Day AS., Ehn M., Gearry RB., Lemberg DA., Leach ST. Fecal S100A12 in healthy infants and children. *Dis Markers* 2013;**35**(5):295–9.

17. Choi BC. Sensitivity and specificity of a single diagnostic test in the presence of work-up bias. *J Clin Epidemiol* 1992;**45**(6):581–6.

Figure legends

Figure 1: CACATU study flow

Figure 2. CACATU study flow from first hospital visit to choice of reference test.

STEP 1: The clinicians registers the patient on the study website www.cacatustudie.eu

STEP 2: The patient (or parent) collects the stool specimen and sends it to the hospital laboratory

STEP 3: The lab divides the specimen in three portions: calprotectin and PCR are immediately performed; one tube is stored at -80 degrees for calgranulin C testing

STEP 4: The lab sends the results of calprotectin and PCR to the researcher.

STEP 5: The researcher enters the test results on the website

STEP 6: The clinician receives a notification with the results and an automated advice on the next best move.

STEP 7: Clinician decides the next best move:

a. In case of high probability of IBD: endoscopy

b. In case of low probability of IBD: follow-up

Table 1: Study inclusion criteria.

One major criterion or two minor criteria are required to classify the patient as "suspected for inflammatory bowel disease".

Major criteria
Persistent diarrhea for more than 4 weeks
Recurrent abdominal pain with diarrhea with at least 2 episodes in the previous 6 months
Rectal blood loss
Peri-anal disease (fistula, deep fissure, abcess)
Minor criteria
Involuntary weight loss
First degree family member with IBD
Anemia (hemoglobin < 2 SD for age and gender)
Increased marker of inflammation (ESR > 20 mm/hour or CRP > 10 mg/L)
Extra-intestinal symptoms (erythema nodosum, arthritis, uveitis, thromboembolism, aphtous ulcera)

Table 2: Data table of principle of paired design for the fecal markers calprotectin and calgranulin C.

Diagnosis: No IBD				
		Fecal Calgranulin C		
		Positive	**Negative**	**Total**
Fecal Calprotectin	**Positive**	Concordant (v)	Discordant (w)	$v + w$
	Negative	Discordant (x)	Concordant (y)	$x + y$
	Total	$v + x$	$w + y$	N_-
Diagnosis: IBD				
		Fecal Calgranulin C		
		Positive	**Negative**	**Total**
Fecal Calprotectin	**Positive**	Concordant (r)	Discordant (s)	$r + s$
	Negative	Discordant (t)	Concordant (u)	$t + u$
	Total	$r + t$	$s + u$	N_+

Null hypothesis H_0 (specificity): $w = x$ Alternative hypothesis H_1: $w \neq x$
Null hypothesis H_0 (sensitivity): $s = t$ Alternative hypothesis H_1: $s \neq t$

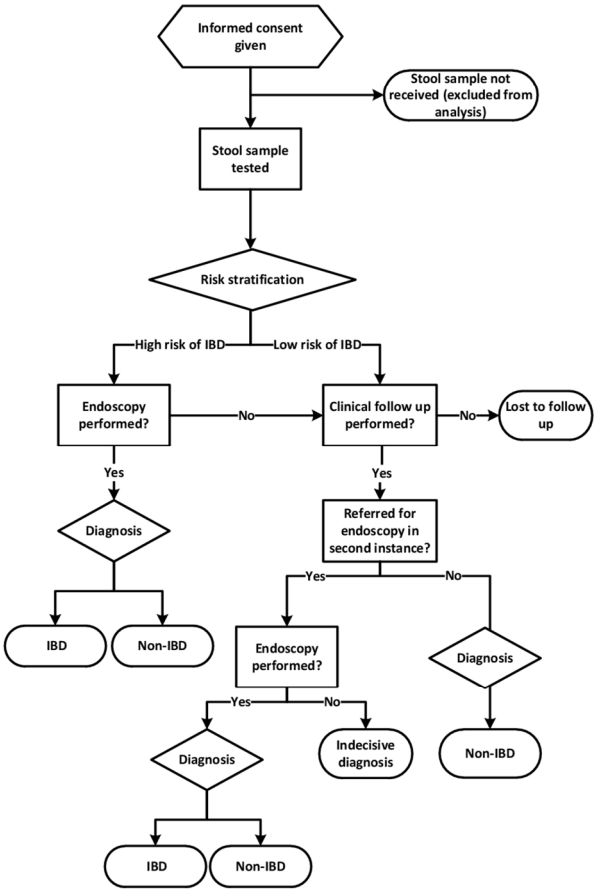

Figure 1: CACATU study flow

209x297mm (300 x 300 DPI)

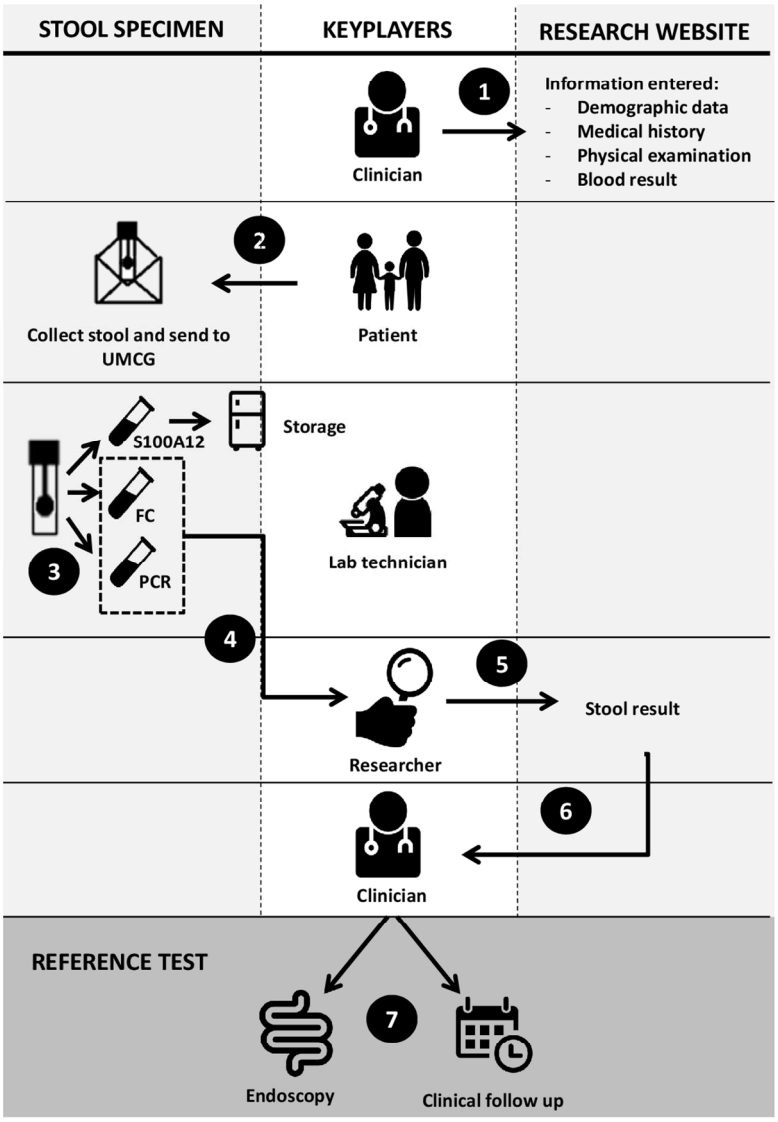

Figure 2. CACATU study flow from first hospital visit to choice of reference test.
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STEP 5: The researcher enters the test results on the website
STEP 6: The clinician receives a notification with the results and an automated advice on the next best move.
STEP 7: Clinician decides the next best move:
a. In case of high probability of IBD: endoscopy
b. In case of low probability of IBD: follow-up

209x297mm (300 x 300 DPI)

SPIRIT 2013 Checklist: Recommended items to address in a clinical trial protocol and related documents*

Section/item	Item No	Description	Addressed on page number
Administrative information			
Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	___1_____
Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry	___1_____
	2b	All items from the World Health Organization Trial Registration Data Set	___n.a._____
Protocol version	3	Date and version identifier	____1_____
Funding	4	Sources and types of financial, material, and other support	____13_____
Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors	____1-13____
	5b	Name and contact information for the trial sponsor	____1_____
	5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities	____13_____
	5d	Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee)	____n.a._____

Introduction			
Background and rationale	6a	Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention	4
	6b	Explanation for choice of comparators	4
Objectives	7	Specific objectives or hypotheses	4
Trial design	8	Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, noninferiority, exploratory)	5
Methods: Participants, interventions, and outcomes			
Study setting	9	Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained	5
Eligibility criteria	10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists)	5
Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	6
	11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving/worsening disease)	n.a.
	11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests)	n.a.
	11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	n.a.
Outcomes	12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended	5
Participant timeline	13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure)	5 (Figure 1&2)

Sample size	14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations	7
Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size	7
Methods: Assignment of interventions (for controlled trials)			
Allocation:			
Sequence generation	16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions	n.a.
Allocation concealment mechanism	16b	Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned	n.a.
Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	n.a.
Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	9
	17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	n.a.
Methods: Data collection, management, and analysis			
Data collection methods	18a	Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol	6,8
	18b	Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols	7

Data management	19	Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol	9
Statistical methods	20a	Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol	10
	20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	10
	20c	Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation)	10
Methods: Monitoring			
Data monitoring	21a	Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed	10
	21b	Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial	n.a
Harms	22	Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct	n.a
Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	n.a.
Ethics and dissemination			
Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	11
Protocol amendments	25	Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC/IRBs, trial participants, trial registries, journals, regulators)	11

Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	11
	26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	11
Confidentiality	27	How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial	9
Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	13
Access to data	29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators	10
Ancillary and post-trial care	30	Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation	n.a.
Dissemination policy	31a	Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions	n.a.
	31b	Authorship eligibility guidelines and any intended use of professional writers	11
	31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	n.a.
Appendices			
Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates	Can be added on request (current version is in Dutch)
Biological specimens	33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable	11

*It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items. Amendments to the protocol should be tracked and dated.

BMJ Open

Selecting children with suspected inflammatory bowel disease for endoscopy with the calgranulin C or calprotectin stool test: protocol of the CACATU study.

Journal:	*BMJ Open*
Manuscript ID	bmjopen-2016-015636.R1
Article Type:	Protocol
Date Submitted by the Author:	19-Mar-2017
Complete List of Authors:	Heida, Anke; Univ Groningen, Pediatric Gastroenterology Vijver, Els; Universitair Ziekenhuis Antwerpen, Pediatric gastroenterology Muller Kobold, Anneke; Univ Groningen, Laboratory Medicine Rheenen, Patrick; Univ Groningen, Pediatric Gastroenterology
Primary Subject Heading:	Gastroenterology and hepatology
Secondary Subject Heading:	Paediatrics
Keywords:	S100A12 protein, S100 proteins, diagnostic accuracy, Inflammatory bowel disease < GASTROENTEROLOGY, Screening

Selecting children with suspected inflammatory bowel disease for endoscopy with the calgranulin C or calprotectin stool test: protocol of the CACATU study.

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Keywords: S100A12 protein; S100 proteins, Inflammatory bowel disease, Screening

Trial registration: ClinicalTrials.gov identifier: NCT02197780 (registered 21 July 2014, last updated 27 August 2016)

Version: 2

Funding: This study is funded by CisBio Bioassays (Codolet, France), developer and producer of the Inflamark® ELISA kit used to measure S100A12 in feces.

Roles and responsibilities: PvR conceived the study. AH, EvdV, AMK and PvR initiated the study design. PvR is the grant holder. All authors contributed to refinement of the study protocol. All authors read and approved the final manuscript.

Cisbio Bioassays did not have a role in the design of this study and will not have any role during its execution, analyses, interpretation of the data, or decision to submit results.

Disclosure of interest: This trial is supported by CisBio Bioassays, producer of the Inflamark® ELISA kit. PvR and AH received financial support from BÜHLMANN Laboratories AG (Schönenbuch, Switzerland) for other ongoing trials.

ABSTRACT:

INTRODUCTION: The introduction of the fecal calprotectin (FC) test to screen children with chronic gastrointestinal complaints has helped the clinician to decide whether or not to subject the patient to endoscopy. In spite of this, a considerable number of patients without IBD is still scoped. Fecal calgranulin C (S100A12) is a marker of intestinal inflammation that is potentially more specific for inflammatory bowel disease (IBD) than FC, as it is exclusively released by activated granulocytes.

OBJECTIVE: To determine whether the specificity of S100A12 is superior to the specificity of FC without sacrificing sensitivity in patients with suspected IBD.

METHODS: An international prospective cohort of children with suspected IBD will be screened with the existing FC stool test and the new S100A12 stool test. The reference standard (endoscopy with biopsies) will be applied to patients at high risk of IBD, while a secondary reference (clinical follow up) will be applied to those at low risk of IBD. The differences in specificity and sensitivity between the two markers will be calculated.

ETHICS AND DISSEMINATION: This study is submitted to and approved by the Medical Ethics Review Committee (MEC) of the University Medical Center Groningen (the Netherlands) and the Antwerp University Hospital (Belgium). The results will be disseminated through a peer-reviewed publication, conference presentation and incorporation in the upcoming National Guideline on Diagnosis and Therapy of IBD in Children.

Trial registration: ClinicalTrials.gov identifier: NCT02197780 (registered 21 July 2014)

STRENGTHS AND LIMITATIONS OF THIS STUDY

- Prospective, multicenter study evaluating the diagnostic accuracy of a new fecal marker (S100A12) with respect to the currently used fecal marker (calprotectin) to select children with gastro-intestinal complaints for endoscopy.
- Our study design reflects current clinical practice in the Netherlands and Belgium.
- Due to the invasive nature of the preferred reference standard (endoscopy) we used clinical follow up as alternative reference test.
- A limitation of the use of two reference standards is the introduction of a differential verification bias.
- We present a Bayesian approach to deal with the introduced differential-verification bias.

INTRODUCTION

Background and rationale

The introduction of the calprotectin stool test to screen children with chronic gastrointestinal complaints has helped the clinician to decide whether or not to refer the patient for endoscopy.[1–4] We have shown that children with normal screening test results (≤ 50 µg/g) have a low probability of inflammatory bowel disease (IBD) and should therefore not undergo the invasive reference test (endoscopy) to exclude IBD.[5] Children with elevated calprotectin levels, however, have a high probability of IBD and require referral to an endoscopy unit for endoscopic evaluation of upper and lower gastrointestinal tract.[1,4,5] Although use of the calprotectin stool test rarely misses a child with IBD, the number of false positive cases who are scoped is considerable.[1,5] Calprotectin is a member of the S100 calcium-binding protein family and is a heterodimer of S100A8 and S100A9. The protein is released mainly by neutrophil granulocytes, but also by other activated and damaged cells including monocytes, macrophages and epithelial cells.[6,7] Calgranulin C (S100A12) is a less investigated member of the S100 protein family.[7,8] Since S100A12 is only released by activated granulocytes, it is suggested to be more specific for gastro-intestinal inflammation caused by IBD than calprotectin.[7,9–11]

Objectives

We hypothesize that a referral strategy based on fecal S100A12 will reduce the number of children wrongly selected for endoscopy as compared to a calprotectin-based strategy. The primary objective is to determine whether the specificity of S100A12 is superior to the specificity of calprotectin without sacrificing sensitivity. The secondary objective is to calculate the diagnostic accuracy characteristics and best cut-offs for both S100A12 and calprotectin.

METHODS

Design

The CACATU study is a prospective, observational, multicenter, diagnostic accuracy study with a paired design. A cohort of children with suspected IBD is screened with the calprotectin stool test (existing test) and with the S100A12 stool test (new test). Confirmation of the target condition (IBD) is based on endoscopy with biopsies (reference standard) or clinical follow up (secondary reference standard).

Study setting

Study participants will be recruited from fifteen general teaching hospitals and one academic center in the Netherlands, and from one general hospital and two academic centers in Flanders, Belgium. The names of all participating centers can be found in the trial registry (www.clinicaltrials.gov). The principal investigators at the various sites are general pediatricians or pediatric gastroenterologists. Six participating centers (3 academic and 3 general hospitals) have a pediatric endoscopy unit.

Eligibility criteria

Patients were eligible if they were between 6 and 17 years old and presented with at least one major criterion or two minor criteria suggestive of IBD (Table 1).

Outcomes

The primary outcome is the difference in specificity between FC and S100A12. Secondary endpoints are the difference in sensitivity and the diagnostic accuracy characteristics (sensitivity, specificity, positive predictive value, negative predictive value, area under the curve, best cut-off point) for both markers individually. All diagnostic accuracy characteristics will be calculated with predefined cut-off points that have been documented in the medical literature, and with best cut-off points based on receiver operator characteristic (ROC) curves.

Intervention

Patients who fulfill the inclusion criteria will be risk-stratified (high vs. low risk of IBD) according to presenting symptoms, blood tests and stool calprotectin. In general we expect that those participants with increased calprotectin levels (>over 50 µg/g) without colon pathogens are likely to be referred to endoscopy (the preferred reference standard) to confirm or exclude IBD. Patients with a normal stool calprotectin test levels are likely to have a low probability of IBD and will be followed clinically to determine the final diagnosis (the alternative reference standard), unless there will be other indications to scope them. Paediatricians will be free to use any diagnostic test, such as celiac disease screening, breath test or ultrasonography (whichever is deemed suitable).

Participant study flow

Eligible participants will be invited for participation by the attending paediatrician. Baseline characteristics, date of birth, major and minor criteria (Table 1), use of NSAIDs and blood tests (hemoglobin, C-reactive protein, erythocyte sedimentation rate, serum alanine transaminase, and gamma-glutamyltranspherase) will be entered on a study website (Figure 2, step 1). Participants will be asked to defecate onto a stool collection sheet held above the toilet water and collect one sample with a screw top container with spoon (step 2). The stool sample is send to the Department of Laboratory Medicine of the University Medical Centre in Groningen (UMCG) in a biomaterial envelope. Immediately after arrival the stool calprotectin level will be measured. The residue will be split with one half stored at -80 degrees Celsius for S100A12 batch testing at a later stage, and the other half will be used to determine enteric pathogens with a Polymerase Chain Reaction (PCR) technique (step 3). The PCR analysis will include Shiga-toxin producing E. Coli, E. Coli O157gen, Cryptosporidium, Dientamoeba Fragilis, Entamoeba histolytica, Giardia Lamblia, Salmonella, Shigella/EIEC and Campylobacter. Results of calprotectin test and PCR analysis will be uploaded on the website, and will then be made visible to the local clinician (step 4 & 5). The paediatrician will receive an e-mail notification with an automated advice on the next best move (step 6). However, the choice of the reference standard (endoscopy or clinical follow up) is up to the paediatrician's discretion (step 7).

Timeline

The process from feces collection to completion of the non-invasive diagnostic work-up is supposed to last no longer than two weeks. We will exclude samples with a transport time that exceeds 7 days and we will perform a sub-analysis with those samples that are received

within 4 days. In case of low risk of relapse the treating pediatricians will receive a reminder for clinical follow up 6 months after inclusion. The total running time of the study is 30 months, including 6 months to complete the follow up.

Sample size

The primary outcome of interest is the difference in specificity between the new test (S100A12) and the established test (FC). If the specificity of S100A12 is superior to the specificity of FC without sacrificing sensitivity, we can replace the old test by the new test. McNemar's test for paired data will be applied to compare specificities between both tests using a 2×2 table exclusively among non-IBD patients (Table 2). Study participants with concordant results ((+,+) or (- ,-)) do not distinguish between the two tests. The only information for comparing the sensitivities and specificities comes from those patients with discordant results ((+,-) or (- ,+)). Sample size calculation is based on recommendations in Hayen et al.[12] Weighed means of specificity of calprotectin were based on a recently published individual patient data meta-analysis.[4] We assumed that fecal S100A12 would lead to a 50% relative improvement of specificity (from 70% to 85%). The prevalence of IBD and non-IBD in the CACATU study cohort is expected to be similar to the prevalence that we found earlier[1], as the study participants will come from the same region and comparable eligibility criteria will apply. The sample size calculation was done with Power Analysis and Sample Size (PASS) software (version 11 for Windows). A sample size of 130 subjects with non-IBD achieves 80% power to detect a difference of 0.15 between the two diagnostic tests whose specificities are 0.70 and 0.85. This procedure used a two-sided McNemar test with a significance level of 0.05. The prevalence of non-IBD in the population is 0.64, and the proportion of discordant pairs is 0.23. We aim to include at least 250 participants, in order to correct for participants diagnosed with IBD (estimated 36%) and participants that will be lost to follow up (estimated 25%).

Recruitment

We asked all participating centers to predict how many eligible patients they could recruit during the enrolment period. Their estimates were based on the list of diagnoses of the previous year, and their estimated totals convinced us that reaching the target sample size is realistic. Retention will be promoted by sending automated reminders to the treating physician to complete the blanks in blood tests, and to re-assess patients with initial low probability of IBD after 6 months.

Test methods

Index tests

Fecal Calprotectin (FC)

FC levels will be measured with the fCal® ELISA test of BÜHLMANN Laboratories AG (Schönenbuch, Switzerland) according to manufacturer's instructions. A level of 50 µg/g is the predefined cut-off value.[2,4,5,13]

Fecal Calgranulin C (S100A12)

S100A12 levels will be measured by one experienced lab technician. The maximal duration of storage of the stool sample in our -80°Celsius freezer is 6 months. Analyses will be performed with a sandwich ELISA, trademark Inflamark® (CisBio Bioassays Codolet, France) on a Dynex DS2 Automated ELISA System (Alpha Labs, Easleigh, UK), according to the manufacturer instructions. In summary, after extraction step, 100 µL of pre-diluted samples will be transferred in duplicate into the corresponding wells coated with anti-S100A12 monoclonal antibody. Incubation time is 30 minutes, followed by three washing cycles with Tween 20. The next step is adding 100 µL of the second monoclonal antibody, anti S100A12 coupled to Horse Radisch-Peroxidase (HRP) followed by a second incubation period of 30 minutes and three washing cycles. Next, 100 µL of the substrate, tetramethyl benzidine, is pipetted in all wells. The wells are protected from light and after 10 minutes, the sulphuric acid stop solution is added. The absorbance will be read at 450 nm. For each duplicate, the mean optical density will be calculated and a calibration curve will be constructed. The curve will be plotted as a cubic regression with DS-matrix software, version 1.23 (Dynex technologies, Chantilly, USA). Purified human S100A12 will be used as calibrator (included in the kit).

The predefined cut-off value of S100A12 is 0.75 mg/kg, which is based on a reference value study among 120 healthy school-aged children and adolescents (pre-liminary, unpublished data).[14]

Reference tests

Endoscopy

Endoscopy will be the reference standard for patients at high risk of IBD. This procedure will be performed under anesthesia by an experienced pediatric gastroenterologist. Ideally, both upper and lower gastrointestinal tract will be evaluated according to the revised Porto criteria[15], and biopsies will be taken from every bowel segment. Histo-pathological examination will be performed by experienced histopathologists. Endoscopists and

histopathologists will have access to clinical information and FC test results, but will be blinded to the results of the S100A12 test.

Clinical follow up

This secondary reference will be applied to patients at low risk of IBD. Six months after study inclusion, the treating paediatrician will receive a notification to enter a second evaluation of major and minor criteria (table 1). Blood tests will only be repeated when deemed necessary by the treating paediatrician. In addition a second feces sample will be collected and send in for analysis. Patients who remain suspected of having IBD will be referred for further investigations in second instance. At study closure one of the researchers (AH) will visit the participating centers to crosscheck patient records for the definite diagnosis.

Rationale for choosing reference standard

Diagnostic endoscopic evaluation of the upper and lower gastrointestinal tract (including intubation of the terminal ileum) in combination with biopsies is the recommended test to diagnose IBD.[15] In children at high risk of IBD with negative endoscopy, small bowel imaging is encouraged.[15] All of these procedures are invasive and require bowel preparation. Endoscopy is mostly performed with the patient under general anesthesia. Although complications are rare, endoscopy is a burdensome procedure for a child. We found it unethical to expose children at low risk of IBD to endoscopy. Therefore, we decided to perform a secondary reference standard (clinical follow up) in patients at low risk for IBD and to adjust for its imperfection.[16]

Blinding

Laboratory personnel will be blinded to the patient's history, and to results of endoscopy and biopsy. Although calprotectin testing is done within 24 hours after arrival of the feces specimen and the residue is stored at -80 degrees Celsius for S100A12 batch testing at a later stage, sample labeling could theoretically link both fecal tests to one patient. Endoscopists and histopathologists will have access to clinical information and calprotectin test results, but will be blinded to the results of the S100A12 test.

Confidentiality and data management

Consecutive patients participating in the study will receive a unique study number. All demographic and medical data will be entered electronically on the study website by the local

investigator and stored linked to this study number. Study investigators will receive access to a secured study website. Local investigators are able to consult only data from participants from their own center. Feces samples will be marked with a study number label and sent to the Department of Laboratory Medicine at the UMCG. Results of calprotectin test and PCR for enteric pathogens will be uploaded on the website by the coordinating investigator and will be visible to the local clinician. At the end of the study the data entered on the study website will be cross-checked with the information in the local Electronic Health Databases. Data will be stored during the study period and until 15 years thereafter. When patients and their parents give permission, residual feces will be stored for a maximum period of 15 years for future diagnostic research. The researchers AH, EvdV and PvR will have access to the final trial dataset.

Statistical methods

Data analysis will be done with SPSS version 22.0 for Windows (SPSS, Chicago, IL, USA). Diagnostic accuracy characteristics (sensitivity, specificity, positive predictive value, negative predictive value) will be presented for both markers individually. McNemar's test for paired data will be applied to compare specificities between both tests using a 2×2 table exclusively among non-IBD patients. Likewise, sensitivities will be compared among IBD cases. We will primarily use pre-specified cut-off points of FC and S100A12. In second instance we will use the best cut-off points based on the receiver operating characteristic (ROC) curves for both FC and S100A12.

We will use a Bayesian correction method to adjust for differential-verification bias in the two reference standards in relation to latent IBD.[17] Based on clinical experience we defined a prior distribution. We assume that our reference standard endoscopy has 95 to 100% sensitivity, and 95 to 100% specificity to diagnose IBD, and that our secondary reference standard clinical follow up will have a sensitivity of 80 to 100%, and a specificity of 60 to 80%. Bayes factors will be calculated using JAGS ('Just Another Gibbs Sampler'), a free program licensed under GNU General Public License.

Finally, we will present the number of true- and false positive and true- and false negative results for four different scenarios: (1) FC screening only; (2) S100A12 screening only; (3) combination of FC and S100A12 in all patients; or (4) combination in a sub-set of patients with inconclusive FC results.

Missing values

In case the index test and reference standard results are missing, the patient will be excluded from further analysis.

Ethical approval & dissemination policy

The study will be conducted according to the principles of the Declaration of Helsinki (59th version, October 2008). The Medical Ethics Review Committee of the University Medical Center Groningen is of the opinion that this study does not require approval according to the Dutch Medical Research Involving Human Subject Act (WMO). The Medical Ethics Review Committee of the Antwerp University Hospital approved the protocol. The legal guardian(s) from all participants, as well as children aged 12 and above, will need to give informed consent for participation and for storage of material for future research.
In case of important protocol amendments, both the Medical Ethics Review Committee and trial registry will be informed. The results of the trial will be disseminated through a peer-reviewed publication, conference presentation and incorporation in the upcoming National Guideline on Diagnosis and Therapy of IBD in Children.

Study status

The first trial participant was included in September 2014. It is anticipated that the trial will end in the spring of 2017.

DISCUSSION

We aim to further improve the accuracy to distinguish patients with a high-risk of IBD from those with a low-risk of IBD with the ultimate goal to reduce the number of futile endoscopies. We will compare the established fecal marker FC with the relatively unknown fecal marker S100A12. The FC test has excellent sensitivity for IBD (0.92 to 0.98),[2–4,18] but its specificity, with point estimates varying between 0.60 and 0.68 [2–4,18] leaves a considerable proportion of non-IBD patients being exposed to an invasive procedure. Studies with fecal S100A12 showed diagnostic promise under ideal testing conditions in pre-selected groups of healthy children and children with IBD.[9,11,19] We only know of one report that compared FC and S100A12 in children presenting with gastrointestinal complaints.[11] The sensitivity and specificity of S100A12 for detection of IBD were both 97%, where FC had a sensitivity of 100% and specificity of 67%.[11]

Methodological biases

In this diagnostic accuracy study the performance of both stool tests will be assessed by verifying the results against endoscopy. Due to the invasive nature of this diagnostic procedure verification can be performed only in a subset of patients with a high risk of IBD. An alternative reference test (i.e. clinical follow up) will be given to the remainder of the patients. The drawback of this so-called differential-verification design is that the second reference test is of lesser quality. Simply adding the results of these two types of reference tests will lead to biased estimates of the overall test accuracy.[16] We plan to correct for this differential-verification bias by using a Bayesian approach, as described by De Groot et al.[17] Secondly, this study is a real life study, in which the decision to expose a child to endoscopy is based on the combination of presenting symptoms, physical examination and results of blood and stool tests, as is currently recommended by Dutch and international scientific societies. Blinding treating physicians for the FC results was therefore irrational and impractical. Knowledge of the FC level will influence the physicians' decision to refer a patient for endoscopic evaluation, which gives rise to a work-up bias.[20] Furthermore, endoscopists will not be blinded for the level of FC and therefore this may theoretically affect the endoscopists' assessment of the endoscopy (diagnostic review bias).

Implications for practice

If S100A12 has a better specificity than FC without sacrificing sensitivity, than S100A12 will be the dominant test to select patients for endoscopy. Replacing FC by S100A12 may then reduce the number of non-IBD patients being subjected to endoscopy. This will be good news for patients (less invasive tests), clinicians (shorter waiting lists for endoscopy), and

health insurance companies (reduction of healthcare cost).

Contributorship statement: PvR conceived the study. AH, EvdV, AMK and PvR initiated the study design. PvR is the grant holder. All authors contributed to refinement of the study protocol. All authors read and approved the final manuscript.
Cisbio Bioassays did not have a role in the design of this study and will not have any role during its execution, analyses, interpretation of the data, or decision to submit results.

Competing interests: This trial is supported by CisBio Bioassays, producer of the Inflamark® ELISA kit. PvR and AH received financial support from BÜHLMANN Laboratories AG (Schönenbuch, Switzerland) for other ongoing trials.

Funding: This study is funded by CisBio Bioassays (Codolet, France), developer and producer of the Inflamark® ELISA kit used to measure S100A12 in feces.

Data sharing statement: Not applicable for this studyprotocol, since we do not present any original data.

References

1. Van de Vijver E, Schreuder AB, Cnossen WR, Muller Kobold AC, van Rheenen PF. Safely ruling out inflammatory bowel disease in children and teenagers without referral for endoscopy. *Arch Dis Child* 2012;**97**(12):1014–8.

2. Holtman GA, Lisman-van Leeuwen Y, Reitsma JB, Berger MY. Noninvasive Tests for Inflammatory Bowel Disease: A Meta-analysis. *Pediatrics* 2016;**137**(1):2015–126.

3. Henderson P, Anderson NH, Wilson DC. The diagnostic accuracy of fecal calprotectin during the investigation of suspected pediatric inflammatory bowel disease: a systematic review and meta-analysis. *Am J Gastroenterol* 2014;**109**(5):637–45.

4. Degraeuwe PLJ, Beld MPA, Ashorn M, Canani RB, Day AS, Diamanti A, et al. Faecal Calprotectin in Suspected Paediatric Inflammatory Bowel Disease: An Individual Patient Data Meta-Analysis. *J Pediatr Gastroenterol Nutr* 2015;**60**:339–46.

5. Heida A, Holtman GA, Lisman-Van Leeuwen Y, Berger MY, Van Rheenen PF. Avoid Endoscopy in Children With Suspected Inflammatory Bowel Disease Who Have Normal Calprotectin Levels. *J Pediatr Gastroenterol Nutr* 2016;**62**:47–9.

6. Røseth AG, Fagerhol MK, Aadland E, Schjønsby H. Assessment of the neutrophil dominating protein calprotectin in feces. A methodologic study. *Scand J Gastroenterol* 1992;**27**(9):793–8.

7. Foell D, Wittkowski H, Roth J. Monitoring disease activity by stool analyses: from occult blood to molecular markers of intestinal inflammation and damage. *Gut* 2009;**58**(6):859–68.

8. Van de Logt F, Day AS. S100A12: a noninvasive marker of inflammation in inflammatory bowel disease. *J Dig Dis* 2013;**14**(2):62–7.

9. De Jong NSH, Leach ST, Day AS. Fecal S100A12: a novel noninvasive marker in children with Crohn's disease. *Inflamm Bowel Dis* 2006;**12**(7):566–72.

10. Kaiser T, Langhorst J, Wittkowski H, Becker K, Friedrich AW, Rueffer A, et al. Faecal S100A12 as a non-invasive marker distinguishing inflammatory bowel disease from irritable bowel syndrome. *Gut* 2007;**56**(12):1706–13.

11. Sidler MA, Leach ST, Day AS. Fecal S100A12 and fecal calprotectin as noninvasive markers for inflammatory bowel disease in children. *Inflamm Bowel Dis* 2008;**14**(3):359–66.

12. Hayen A, Macaskill P, Irwig L, Bossuyt P. Appropriate statistical methods are required to assess diagnostic tests for replacement, add-on, and triage. *J Clin Epidemiol* 2010;**63**(8):883–91.

13. Kostakis ID, Cholidou KG, Vaiopoulos AG, Vlachos IS, Perrea D, Vaos G. Fecal calprotectin in pediatric inflammatory bowel disease: a systematic review. *Dig Dis Sci* 2013;**58**(2):309–19.

14. Heida A, Kobold A, Wagenmakers L, Van De Belt K, Van Rheenen P. Reference values of fecal calgranulin C (S100A12) in school aged children and teenagers. *Journal of Pediatric Gastroenterology and Nutrition*, vol. 62. 2016. p. Suppl 1 (17).

15. Levine A, Koletzko S, Turner D, Escher JC, Cucchiara S, de Ridder L, et al. ESPGHAN revised porto criteria for the diagnosis of inflammatory bowel disease in children and adolescents. *J Pediatr Gastroenterol Nutr* 2014;**58**(6):795–806.

16. Naaktgeboren CA, de Groot JAH, Rutjes AWS, Bossuyt PMM, Reitsma JB, Moons KGM. Anticipating missing reference standard data when planning diagnostic accuracy studies. *BMJ* 2016;**352**:i402.

17. De Groot JAH, Dendukuri N, Janssen KJM, Reitsma JB, Bossuyt PMM, Moons KGM. Adjusting for differential-verification bias in diagnostic-accuracy studies: a Bayesian approach. *Epidemiology* 2011;**22**(2):234–41.

18. Lin J-F, Chen J-M, Zuo J-H, Yu A, Xiao Z-J, Deng F-H, et al. Meta-analysis: fecal calprotectin for assessment of inflammatory bowel disease activity. *Inflamm Bowel Dis* 2014;**20**(8):1407–15.

19. Day AS, Ehn M, Gearry RB, Lemberg DA, Leach ST. Fecal S100A12 in healthy infants and children. *Dis Markers* 2013;**35**(5):295–9.

20. Choi BC. Sensitivity and specificity of a single diagnostic test in the presence of work-up bias. *J Clin Epidemiol* 1992;**45**(6):581–6.

Figure legends

Figure 1: CACATU study flow

Figure 2. CACATU study flow from first hospital visit to choice of reference test.

STEP 1: The clinicians registers the patient on the study website www.cacatustudie.eu

STEP 2: The patient (or parent) collects the stool specimen and sends it to the hospital laboratory

STEP 3: The lab divides the specimen in three portions: calprotectin and PCR are immediately performed; one tube is stored at -80 degrees for calgranulin C testing

STEP 4: The lab sends the results of calprotectin and PCR to the researcher.

STEP 5: The researcher enters the test results on the website

STEP 6: The clinician receives a notification with the results and an automated advice on the next best move.

STEP 7: Paediatrician decides the next best move:

a. In case of high probability of IBD: endoscopy*

b. In case of low probability of IBD: clinical follow-up

* The ultimate decision to scope is in the hands of the endoscopist

Table 1: Study inclusion criteria.

One major criterion or two minor criteria are required to make the patient eligible for participation in the CACATU study.

Major criteria
Persistent diarrhea for more than 4 weeks
Recurrent abdominal pain with diarrhea with at least 2 episodes in the previous 6 months
Rectal blood loss
Peri-anal disease (fistula, deep fissure, abcess)
Minor criteria
Involuntary weight loss
First degree family member with IBD
Anemia (hemoglobin < 2 SD for age and gender)
Increased marker of inflammation (ESR > 20 mm/hour or CRP > 10 mg/L)
Extra-intestinal symptoms (erythema nodosum, arthritis, uveitis, thromboembolism, aphtous ulcera)

Table 2: Data table of principle of paired design for the fecal markers calprotectin and calgranulin C.

Diagnosis: No IBD				
		Fecal Calgranulin C		
		Positive	**Negative**	**Total**
Fecal Calprotectin	**Positive**	Concordant (v)	Discordant (w)	$v + w$
	Negative	Discordant (x)	Concordant (y)	$x + y$
	Total	$v + x$	$w + y$	N_-
Diagnosis: IBD				
		Fecal Calgranulin C		
		Positive	**Negative**	**Total**
Fecal Calprotectin	**Positive**	Concordant (r)	Discordant (s)	$r + s$
	Negative	Discordant (t)	Concordant (u)	$t + u$
	Total	$r + t$	$s + u$	N_+

Null hypothesis H_0 (specificity): $w = x$ Alternative hypothesis H_1: $w \neq x$
Null hypothesis H_0 (sensitivity): $s = t$ Alternative hypothesis H_1: $s \neq t$

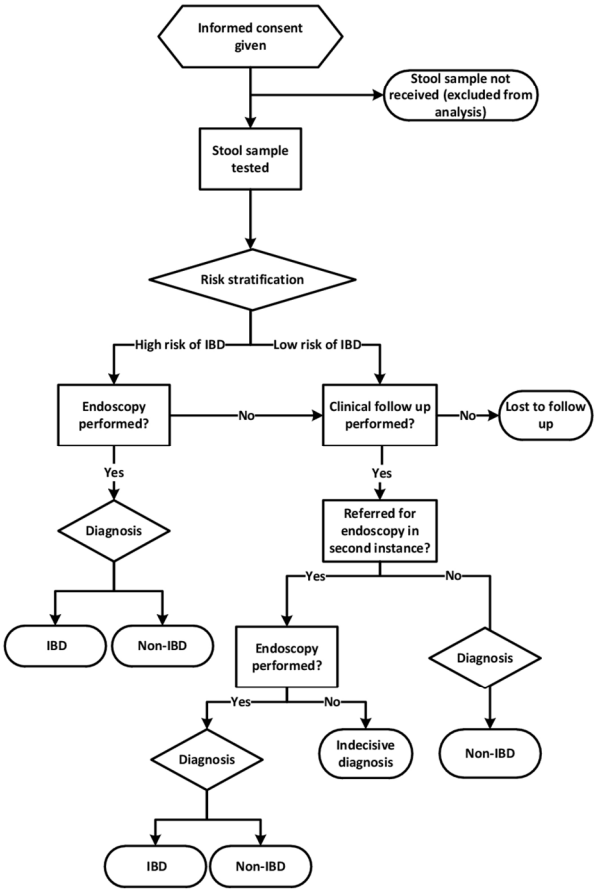

Figure 1: CACATU study flow

209x297mm (300 x 300 DPI)

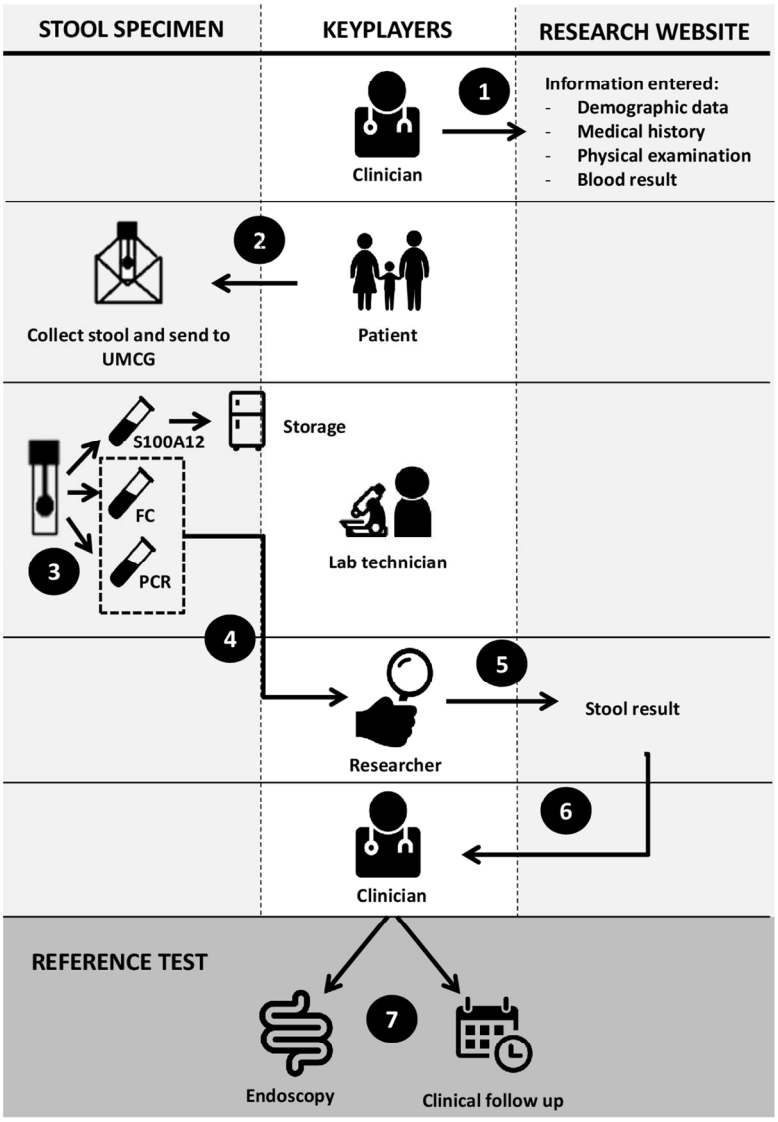

Figure 2. CACATU study flow from first hospital visit to choice of reference test.
STEP 1: The clinicians registers the patient on the study website www.cacatustudie.eu
STEP 2: The patient (or parent) collects the stool specimen and sends it to the hospital laboratory
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STEP 4: The lab sends the results of calprotectin and PCR to the researcher.
STEP 5: The researcher enters the test results on the website
STEP 6: The clinician receives a notification with the results and an automated advice on the next best move.
STEP 7: Clinician decides the next best move:
a. In case of high probability of IBD: endoscopy
b. In case of low probability of IBD: follow-up

209x297mm (300 x 300 DPI)

SPIRIT 2013 Checklist: Recommended items to address in a clinical trial protocol and related documents*

Section/item	Item No	Description	Addressed on page number
Administrative information			
Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	___1_____
Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry	___1_____
	2b	All items from the World Health Organization Trial Registration Data Set	___n.a._____
Protocol version	3	Date and version identifier	____1_____
Funding	4	Sources and types of financial, material, and other support	____13_____
Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors	____1-13____
	5b	Name and contact information for the trial sponsor	____1_____
	5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities	____13_____
	5d	Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee)	____n.a._____

Introduction			
Background and rationale	6a	Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention	4
	6b	Explanation for choice of comparators	4
Objectives	7	Specific objectives or hypotheses	4
Trial design	8	Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, noninferiority, exploratory)	5
Methods: Participants, interventions, and outcomes			
Study setting	9	Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained	5
Eligibility criteria	10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists)	5
Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	6
	11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving/worsening disease)	n.a.
	11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests)	n.a.
	11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	n.a.
Outcomes	12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended	5
Participant timeline	13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure)	5 (Figure 1&2)

Sample size	14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations	7
Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size	7
Methods: Assignment of interventions (for controlled trials)			
Allocation:			
Sequence generation	16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions	n.a.
Allocation concealment mechanism	16b	Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned	n.a.
Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	n.a.
Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	9
	17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	n.a.
Methods: Data collection, management, and analysis			
Data collection methods	18a	Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol	6,8
	18b	Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols	7

Data management	19	Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol	9
Statistical methods	20a	Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol	10
	20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	10
	20c	Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation)	10
Methods: Monitoring			
Data monitoring	21a	Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed	10
	21b	Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial	n.a
Harms	22	Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct	n.a
Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	n.a.
Ethics and dissemination			
Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	11
Protocol amendments	25	Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC/IRBs, trial participants, trial registries, journals, regulators)	11

Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	11
	26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	11
Confidentiality	27	How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial	9
Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	13
Access to data	29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators	10
Ancillary and post-trial care	30	Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation	n.a.
Dissemination policy	31a	Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions	n.a.
	31b	Authorship eligibility guidelines and any intended use of professional writers	11
	31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	n.a.
Appendices			
Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates	Can be added on request (current version is in Dutch)
Biological specimens	33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable	11

*It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items. Amendments to the protocol should be tracked and dated.