CONSORT 2010 checklist of information to include when reporting a randomised trial*

Title and abstract

1a	The Effects of Oral Proteolytic and Hydrolytic Enzymes and Flavonoids on Inflammatory Markers and Coagulation after Marathon Running Short title: „Enzy-MaGIC“-Study: Marathonstudie 2013 (Enzymes, Marathon runninG, Inflammation, Coagulation-Study)
1b	BACKGROUND: Regular moderate intensity physical activity positively influences the immune system with a lower incidence of upper respiratory tract infections (URTI) and lower levels of pro-inflammatory markers. However, marathon running due to its strenuous and prolonged nature results in immune perturbations with a major increase in pro-inflammatory markers and subsequent increased incidence of URTI. Furthermore, marathon running results in muscle damage and changes in hemostasis that promote a pro-thrombotic state. Naturally occurring hydrolytic enzymes and flavonoids have antioxidant, anti-inflammatory and fibrinolytic effects, and may serve as countermeasures to exercise-induced inflammation, immune dysfunction and URTI.The aim of this study is to determine whether the ingestion of oral hydrolytic enzymes and flavonoids before and after a marathon attenuates post-race muscle damage and inflammation, counters pro-thrombotic changes in hemostasis and decreases URTI incidence. METHODS/DESIGN: The Enzy-MagIC-study (Enzymes, Marathon runninG, Inflammation, Coagulation) is a randomized, double-blind, placebo-controlled, monocenter phase I trial. 160 healthy males (age 20-65 years) will be randomized to receive either placebo or treatment (Wobenzym, MUCOS Pharma, Berlin, Germany) which contains the hydrolytic enzymes (bromelain, trypsin) and the flavonoid rutoside. One week before the marathon race, participants will begin daily ingestion of the investigational product (3×4 tablets). Intake will be continued for two weeks after the race (3×2 tablets per day). Clinical and laboratory measures will be collected 5-weeks and 1-week before the race, and immediately-, 24-h, 72-h, and 2 weeks after the race. The primary endpoint is the influence of the treatment on the pre-to-post marathon race plasma concentration change of the inflammatory marker interleukin-6 (IL-6). Secondary endpoints include the effect of treatment on salivary IgA concentration and the frequency of upper respiratory tract infections (URTI) for two weeks post-marathon as determined by the Wisconsin Upper Respiratory Symptom Survey (WURSS-24). Furthermore, changes of muscular and rheological parameters will be measured before and after the marathon race.

		DISCUSSION: We hypothesize that marathon-induced inflammatory perturbations and the incidence of subsequent URTI, muscular damage, and changes of hemostasis can be positively influenced by the anti-edematous, anti-inflammatory, antioxidant, and fibrinolytic effects of oral hydrolytic enzymes and flavonoids (Wobenzym).
Introduction		
Background and objectives	2a	The influence of extreme physical stress, such as that experienced during marathon running, on the inflammatory system and coagulation has been investigated in recent studies. However, up until now, countermeasures against these adverse effects have not been adequately identified. Immune-modulators (e.g. flavonoids) are known to be present in fruits and vegetables. There are also several different proteolytic and hydrolytic enzymes found in fruits most of whom are attributed health-protective properties. Therefore, 160 trained and untrained runners will consume a mixture of different enzymes and flavonoids (Wobenzym® plus) or placebo and effects on changes in inflammation and hemostasiologic parameters will be investigated during and after extreme physical stress (participation in a marathon race). The clinical result of the perturbation of the immune system will be assessed by the Wisconsin Upper Respiratory Symptom Survey (WURSS-24) with regard to symptoms of the upper respiratory tract system.
	2b	It is hypothesized that the intake of a product with potential immunomodulatory effects (Wobenzym® plus, consisting of proteolytic and hydrolytic enzymes and flavonoids) can positively influence both inflammation and blood coagulation pertubations which are usely observed after a marathon race. The present study investigates changes in inflammatory status and incidence of URTI after extreme aerobic physical stress (participation in a marathon race). In addition, the impact of marathon running on the hemostasis and muscular state will be evaluated. Changes at the inflammatory, muscular, and rheological level will be related to ingestion of oral hydrolytic enzymes and bioflavonoids.
Methods		
Trial design	3a	Enzy-Magic was a prospective randomized, double-blind, placebo-controlled, and monocenter phase I trial. In 160 athletes we test the effects of a marathon run on cardiac function and inflammation after the intake of oral proteo-/hydrolytic enzymes and bioflavonoids (Wobenzym® plus). Participants were randomly assigned in a 1:1 manner to receive either Wobenzym Plus (MUCOS Pharma, Berlin, Germany, WOB) or placebo (PL).
	3b	There were no important changes to methods after trial commencement.
Participants	4a	- Healthy male, Age 20-65 years, - History of at least one successfully finished half marathon, - Intention to participate at the Munich Marathon 2013, - Subject is able to read, understand, and sign a written Informed Consent to participate in the Enzy-MagIC-Study

	4b	Department of Prevention, Rehabilitation and Sports Medicine, Klinikum rechts der Isar, Technische Universitaet Muenchen, Munich, Germany
Interventions	5	One week before the marathon race, the participants began daily ingestion of WOB (3x4 tablets up to the marathon race) and for the two weeks after the marathon race 3x2 tablets. WOB is an orally applied enzyme-rutoside combination supplement. Each tablet contains 90 mg bromelain (450 FIP units), 48 mg trypsin, and 100 mg rutoside. Both WOB dosages prior and after the race are in accordance with recommendations in the patient information sheet. In case of heavy inflammatory illnesses or after acute trauma, the recommended dosage is 12 tablets (correspondingly 1080 mg bromelain (5400 FIP units), 576 mg trypsin, and 1200 mg rutoside) per day. Therefore, 12 tablets per day prior to the race served as the loading dose before the marathon race. After this loading period, the dosage was decreased to a maintenance dosage of 6 tablets per day.
Outcomes	6a	The primary endpoint criterion is the concentration change of the inflammatory marker Interleukin-6 (IL-6) before to after a marathon in relation to the consumption of oral hydrolytic enzymes and bioflavonoids (Wobenzym® plus). The secondary endpoints include the effect of consuming oral proteo-/hydrolytic enzymes and bioflavonoids (Wobenzym® plus) on the frequency of upper respiratory tract infections (URTI) post-marathon and changes in muscular, rheological parameters and muscular strain. Participants were screened to assess inclusion and exclusion criteria within 5 weeks before the marathon (visit 1, V1). Baseline data were collected, including laboratory measurements (IL-6, muscular, rheological parameters), electrocardiogram, physical examination, anthropometry, training history questionnaires, echocardiography, ultrasound of the carotid arteries and exercise testing with spiroergometry. Laboratory measurements were collected immediately (visit 2), 24-h (visit 3) and 72-h (visit 4) after the race. One week prior to the race and during the 2-week period following the race URI-rates were assessed daily by the Wisconsin Upper Respiratory Symptom Survey (WURSS-24).
	6b	No changes to trial outcomes after the trial commenced.
Sample size	7a	Sample size calculation was based on the primary endpoint criterion, the difference in marathon-induced change of plasma IL-6 between the study groups. Due to the expected right-skewed distribution of IL-6, differences in changes of logarithmized IL-6 levels (relative group changes) were considered as the base for inferential statistics. From previous publications a coefficient of variation (CV) of 60% for IL-6 changes was expected for the proposed study population. As a clinically relevant relative difference of 25% between the groups was assumed, a sample size of 80 individuals per group was planned to be included in order to detect a difference in IL-6 changes between both groups (given the CV of 60%) with a power of 90% on a two-sided level of significance of 0.05 using a two-sample t-test.
Randomisation:		

Sequence generation	8a	A randomization list was generated by an independent statistician of the Institut für Medizinische Statistik und Epidemiologie (IMSE) der Technischen Universität München using the software Rancode 3.6 Professional.
	8b	Block randomization was used and an equal number of individuals was planned to be allocated to the verum and to the placebo group. The listing showed the output of the randomization software that was concealed until the unblinding of the treatment allocation. The listing showed the center in the first column (only one center was included), the participants number ("Probandennummer") in the second column and the assigned treatment showing a numerical code (1 = Verum, 2 = Placebo) and the name of the assigned treatment.
Allocation concealment mechanism	9	The randomization list was sent to MUCOS Pharma, Berlin, Germany. According to the randomization list they sent us sequentially numbered containers back. Participant received these containers according to their participant's number.
Implementation	10	A randomization list was generated by an independent statistician of the Institut für Medizinische Statistik und Epidemiologie (IMSE) der Technischen Universität München Investigators of the Department of Prevention, Rehabilitation and Sports Medicine, Klinikum rechts der Isar, Technische Universitaet Muenchen, Munich, Germany enrolled participants Principal investigator Dr. med. Johannes Scherr assigned participants to interventions
Blinding	11a	After assignment to interventions the principal investigator, the investigators and the participants were blinded (see also 9).
Statistical methods	12a	Statistical methods used to compare groups for primary and secondary outcomes Primary analysis: Changes of logarithmic IL6 values from Visit 1 to Visit 3 (ln(IL6post) – ln(IL-6pre)) were compared between both groups. A two-sided t test for independent samples using Satherthwaite's approximation to allow for different variances in both study groups was used for analysis of the primary endpoint. A level of significance of 0.05 was used. Means and standard deviations for both groups are shown and a 95% confidence interval for the difference in means is presented. Primary analysis was performed according to the intention-to-treat (ITT) principle. The full analysis set (FAS) consists of all participants that took at least one dose of study medication and have a valid IL-6 assessment at Visit 1. An imputation strategy for missing values that cannot artificially favour the verum group (conservative imputation) was applied for the primary analysis. For subjects in the verum group who did not start the marathon, who did not run at least 30 kilometers in the mara-thon, or who have no valid IL-6 assessment after the marathon, the highest IL-6 value that was observed in any individual after the marathon was imputed. For individuals in the placebo group, missing values were replaced by the lowest observed value of all individu-als that finished the marathon. Various imputation strategies (multiple imputation, worst case scenario for both groups) and use of

different analysis sets (per protocol set, complete cases only) were considered as sensitivity analyses. The per protocol set consists of all individuals that finished the marathon, have valid IL-6 assessments at visit 3, took between 80% and 125% of the allocated medication, and did not take any NSAID during the study. All imputation strategies and analysis sets were defined before unblinding the data.

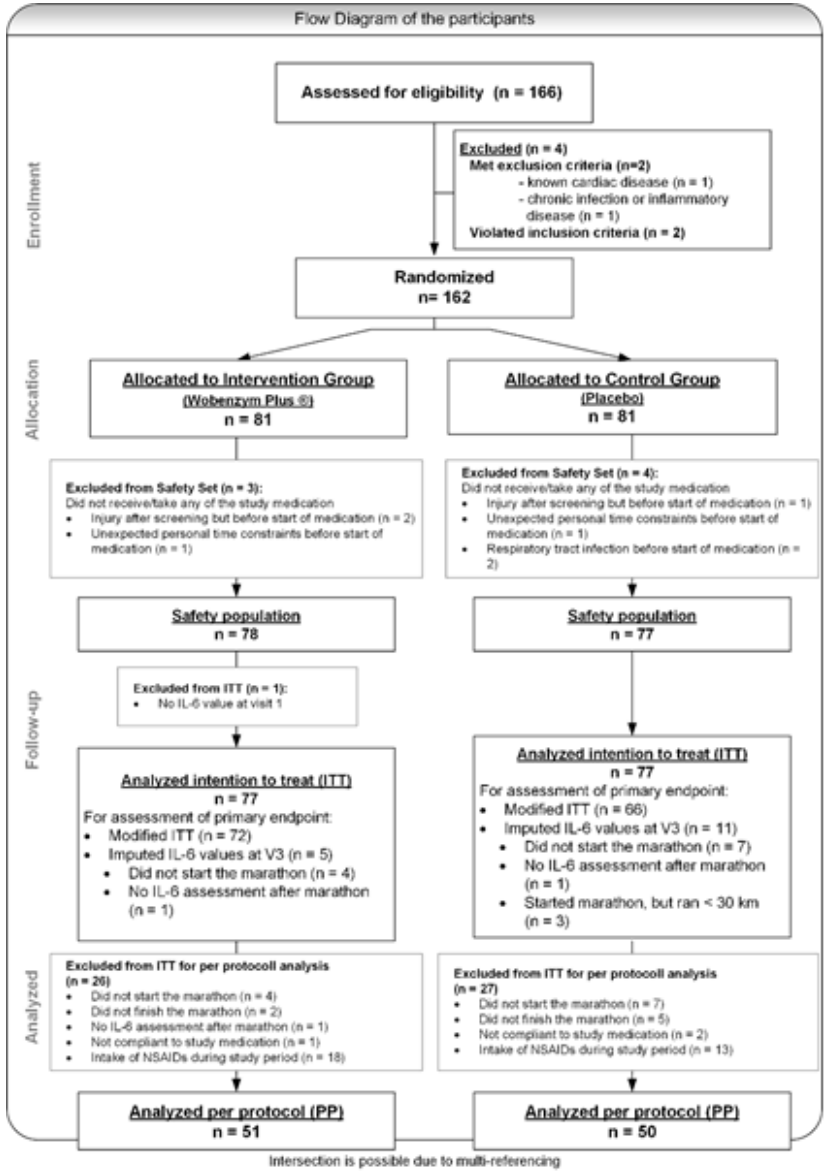
Secondary endpoints were analysed in an explorative manner. For all secondary endpoints only complete observations of individuals who ran at least 30 kilometers of the marathon were considered.
For inflammation markers group comparisons were performed using differences of logarithmic post and pre marathon values as described for the primary endpoint.
For group comparisons of changes in cardiac, muscular, and rheological parameters changes from Visit 1 to Visit 3 were compared between groups using two-sided independent samples t-test with Satterthwaite's approximation. Observed means and standard deviations of changes are presented for both groups and a 95% confidence interval for the difference of means was estimated.
Upper respiratory tract infections (URTI) were identified from the Wisconsin Upper Respiratory Symptom Survey (WURSS). Subjects with a sum score of at least 7 for WURSS question 2 to 23 were defined as having an URTI. Incidence rates of URTI post marathon was compared between both groups using a X2 test, by comparing the proportion of subjects with at least one post marathon assessment with a WURSS score of at least 7. Additionally, for each individual the mean and the maximum post marathon WURSS score was determined and these were compared between both study groups with a two-sided Wilcoxon rank sum test, as a skewed distribution of these measures was detected in the blinded data review.
For assessment of safety, descriptive statistics (mean, standard deviation, minimum, median, and maximum) are presented for laboratory data and for vital signs for all visits. Crosstables providing the number of individuals with laboratory data outside the predefined reference ranges are given. All observed adverse events are listed and summaries for observed adverse events for both study groups are given. All subjects who took at least one dose of the study medication are included in the Safety Set used for assessment of safety.

12b Methods for additional analyses, such as subgroup analyses and adjusted analyses
Subgroups: Analysis of the primary endpoint was repeated for predefined relevant subgroups depending on age (20 to 40 years, older than 40 years), BMI (less than 20 kg/m2, 20-25 kg/m2) more than 25 kg/m2), marathon time (below 3:30 h, 3:30 to 4:00 h, more than 4:00 h), and on the numbers of kilometers ran during the training phase per week (quartiles). Quartiles of the numbers of kilometers ran during the training phase were determined in the blinded data review to be less than 35 kilometers, 35 to 50 kilometers, 50 to 65 kilometers and more than 65 kilometers per week.

Additional analyses: Due to the extreme values (outlier) which had to be used in the imputation mentioned above (12a), we additionally performed the analysis of the primary endpoint in a modified ITT (mITT) and per protocol set (with the intention to present an estimate displaying the treatment effect observed in reality). The mITT set included all individuals who ran at least 30 kilometers of the marathon and who had valid IL-6 assessments at baseline and immediately after the marathon. The per protocol set consists of all individuals that finished the marathon, have valid IL-6 assessments at visit 3, took between 80% and 125% of the allocated medication, and did not take any NSAID during the study period. No imputation was performed and participants with missing values were excluded from the analysis. By this, a better estimate for the real treatment effect should be provided; however control of the type I error could not be guaranteed a priori due to possibly informative drop-outs and missing values. Analysis of the primary endpoint was conducted with the ITT, mITT and per protocol set.

Results

Participant flow (a diagram is strongly recommended) 13a,b



Recruitment 14a Study initiation date: 03.09.2013, Study completion date 05.02.2014

14b completed

Baseline data 15 **Baseline characteristics of the study participants (ITT set)**

	WOB [n=77]	**PL [n=77]**
Anthropometry		
Age [yrs]	44 ± 10	40 ± 11
BMI [kg/m²]	23.2 ± 2.0	23.4 ± 2.4
Body fat [%]	13.5 ± 4.1	13.2 ± 4.7
Systolic blood pressure [mmHg]	123 ± 11	124 ± 12
Marathon run		
Marathon time [h:mm]	3:52 ± 0:36	3:41 ± 0:37
Minimum/Maximum race time [h:mm]	2:20 / 5:13	2:38 / 6:02
Exercise intensity during race [%HRmax]	85 ± 7	86 ± 5
Training history		
Training kilometers during the last 10	56 ± 25	58 ± 20
Number of finished marathon races	4 [0-100]	3 [0-45]
Cardiovascular risk factors (%)		
Family history of CVD	16	13
Smoking/ ex-smoking	3 / 1	1 / 3

Data are presented as means ± SD, median [min-max] or %

Numbers analysed 16 Analysis of the primary endpoint was performed following the intention-to-treat (ITT) principle. The ITT dataset consists of all trial subjects enrolled into the trial and randomized (n=154). We additionally performed the analysis of the primary endpoint in a modified ITT (mITT) (n=138) and per protocol set (n=101). All safety issues were assessed in the safety set including all patients who took at least one dose of their study medication (n=155). All analysis was by original assigned groups.

Outcomes and estimation 17a Immediately post-race, a significant difference in increase of IL-6 between the WOB and PL group in the ITT set after conservative imputation for the WOB group and liberal imputation for the PL group was observed (median [IQR]: WOB: 35.2 [72.2-591.0] ng/L vs. PL: 33.3 [49.5-133.4] ng/L, p = 0.003). There was no significant difference in increase of IL-6 between groups in the mITT set (median [IQR]: WOB: 33.8 [22.5-58.8] ng/L vs. PL: 35.6 [24.8-61.29] ng/L, p = 0.758) and in the PP set (median [IQR]: WOB: 35.4 [72.2-591.0] ng/L vs. PL: 35.1 [63.2-124.0] ng/L, p = 0.292). Plasma IL-6 level of the mITT did not differ significantly between groups at all other laboratory visits.

Secondary Outcome

URI:

On 136 of 1070 documented days (12.7 %) an URI was observed in the WOB group, URI was detected

on 157 of 991 reported days in the PL group (15.8 %). From race day until 2 weeks after the marathon race, the percentage of individuals with at least one URI did not significantly differ between the groups (WOB: 50.0 %, PL: 51.5 %, p = 0.859).

Blood leukocyte counts, hs-CRP and IL-10:

Immediately post-race, there was no significant difference in increase of blood leukocyte counts and IL-10 between groups (mean ± SD: leukocytes: WOB: 11.0 ± 4.1x109/L, PL 10.5 ± 3.0x109/L; p = 0.862; IL-10: WOB: 83.9 ± 105.6 mg/L, PL: 57.2 ± 49.5 mg/L; p = 0.550). Hs-CRP value changes pre-to-post race showed no significant group differences with an increase of hs-CRP level 72 hours after the race (mean ± SD: WOB: 12.55 ± 9.6 mg/L, PL: 10.1 ± 12.8 mg/L; p = 0.102).

Ancillary analyses 18

Subgroup analysis of IL-6 Increase pre-to-post marathon [ng/L] for modified ITT

Training [km/week]	**Participants**	**IL-6 Increase**	**IL-6 Increase**	**P Value**
≥ 65	36	82.0 ± 129.1	67.1 ± 37.8	0.738
50-65	38	33.5 ± 18.6	42.8 ± 23.7	0.163
35-50	32	62.6 ± 52.8	35.3 ± 20.1	0.061
< 35	32	38.3 ± 29.4	33.3 ± 25.9	0.454
Age [years]				
> 40	82	65.1 ± 106.1	44.9 ± 29.0	0.784
20-40	56	47.8 ± 42.2	45.9 ± 31.6	0.377
BMI [kg/m²]				
> 25	33	33.4 ± 12.4	51.6 ± 48.6	0.222
20-25	101	45.9 ± 35.5	48.1 ± 32.5	0.703
≤ 20	4	591.0	72.6 ± 48.5	-
Marathin finishing				
> 4:00	41	30.8 ± 21.7	33.7 ± 15.1	0.526
3:30-4:00	50	51.0 ± 31.1	38.0 ± 26.6	0.031
< 3:30	47	93.9 ± 131.8	56.4 ± 33.5	0.524

Harms 19

Adverse events (AE): A total of 66 participants (43%) out of 155 participants of the safety population suffered at least one AE during treatment period. The percentage of participants with at least one AE did not differ significantly between groups with 46 % of the participants in the WOB group and 39 % PL group (p=0.368). Headache was most frequently reported; 9%, followed by knee pain (8%) and diarrhoea (5%). Severe adverse events were observed in 4 subjects (6 %). Severe adverse events (judgement to relation to study medication) were erythema (possible) and hearing loss (unlikely) in the PL group as well as erythema (possible) and bleeding nose (unlikely) in the WOB group. For 44% of the subjects with an AE (n=29), the AE was judged by the investigator to be related to the study medication (mainly diarrhoea

		and headache). All other AEs were classified as not related to the study medication. Relevant laboratory findings: There were no clinically significant laboratory findings exceeding physiological responses to strenuous and prolonged exercise.
Discussion		
Limitations	20	First, we used a conservative imputation strategy for our primary endpoint that would not favor the WOB group. There was one athlete in the WOB group with an unexpected high IL-6 level after the race (591 ng/L; 6.5-times higher compared to mean in WOB group and 3.1-times higher than observed in our prior trials which has been used for imputation analyses in ITT according to our statistical analysis plan. Using the modified ITT set without imputation or the per protocol set, the significantly lower increase of IL-6 in the PL group as compared to the WOB group, which was supported by our imputation strategy, was not seen. The conservative imputation strategy displayed a result which overweighed the outlier value and therefore we decided to present the results of the primary end point in all three data sets (ITT, mITT and per protocol) and for the secondary endpoints on the basis of a modified ITT set (as predefined). Second, we did not measure the amounts of quercetin, trypsin and bromelain absorbed from the intestinal tract and plasma concentrations. Nonetheless, bioavailability of the supplement used in this study has been tested in humans, and much smaller doses resulted in increased plasma levels of these metabolites with a varying elimination half-life within a range of 5-48 hours. Thus, the expected anti-inflammatory effect directly after the marathon race was not limited by the dosing amount and regimen before and after the marathon race. Furthermore, possible mechanisms of interference on absorption (e.g. anti-inflammatory medication, food) were minimized so that other possible explanations for the results of our study are unlikely.
Generalisability	21	Despite these limitations, this large, prospective randomized, double-blind, placebo-controlled Phase I trial in non-elite male athletes had high statistical power (90%), allowing reliable conclusions to be drawn from the data.
Interpretation	22	Supplementation with rutoside and hydrolytic enzymes for one week prior to marathon running did not attenuate post-race inflammation. Continued supplementation during the 2-week period after the race could not reduce the incidence of clinically relevant URI. There is a growing realization that the bioactive influences of individual flavonoids are potentiated when mixed with other flavonoids, or included in a cocktail or extract of other polyphenols and nutrients. Research is needed to define better optimal dosing regimens and whether unique flavonoid mixtures that include several of the most bioactive flavonoids across different subgroups amplify anti-inflammatory influences, while also bolstering immunity and countering post-race URI
Other information		
Registration	23	ClinicalTrials.gov (NCT01916408)

		Ethics committee of the University Hospital Klinikum rechts der Isar, Munich, Germany approval reference number, 5820/13 Federal Institute for Drugs and Medical Devices, Germany (approval reference number, 4039219)
Protocol	24	Grabs V, Nieman DC, Haller B, Halle M, Scherr J: The effects of oral hydrolytic enzymes and flavonoids on inflammatory markers and coagulation after marathon running: study protocol for a randomized, double-blind, placebo-controlled trial. BMC Sports Sci Med Rehabil 2014, 6: 8
Funding	25	Funding for the study was partly received by MUCOS Pharma GmbH. The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

*We strongly recommend reading this statement in conjunction with the CONSORT 2010 Explanation and Elaboration for important clarifications on all the items. If relevant, we also recommend reading CONSORT extensions for cluster randomised trials, non-inferiority and equivalence trials, non-pharmacological treatments, herbal interventions, and pragmatic trials. Additional extensions are forthcoming: for those and for up to date references relevant to this checklist, see www.consort-statement.org.