TITLE PAGE

Full Title:

Systemic Effect of Lycosome-Formulated Dark Chocolate and GA Lycopene on Gut Microbiota, Liver Metabolism, Blood Markers of Oxidative Status and Skin Parameters in Healthy Overweight Volunteers

***Protocol Number*: DT 04---11---29---18**

Clinical Trial Phase: **Phase two**

Principal Investigator (PI): **Ivan M Petyaev MD, PhD**

Funding Mechanism: **Sponsored by Lycotec Ltd**

Protocol Chair/Co---Chair: **Yuriy K Bashmakov MD, PhD, Nigel H Kyle**

Medical Officer/Medical Monitor: **Victor Klochkov MD, PhD, Yuriy K Bashmakov MD, PhD**

Executive Secretary: **Natalia Chalyk PhD**

Investigational compound / nutraceuticals: Lycosome-formulated Dark Chocolate, GA Lycopene (Lycotec Ltd, UK)

SIGNATURE PAGE

We agree to conduct the study in accordance with the relevant, current protocol and will not make changes to the protocol without permission of the funding authority, except when necessary to protect the safety, rights, or welfare of study participants.

We agree to personally conduct or supervise this study.

We will ensure that the requirements relating to obtaining informed consent and Ethics Committee (EC) or Institutional Review Board (IRB) review are met.

We agree to report to the sponsor any adverse experiences that occur during the course of this study.

We agree to maintain adequate and accurate study records and to make those records available for inspection by any authorized representatives, and/or other applicable regulatory entities. We will ensure that an EC or IRB that complies with the requirements of Ethics in Conduct of Research will complete initial and continuing review and approval of the study. We also agree to promptly report to the EC/IRB all changes to the study and all unanticipated problems involving risks to human subjects or others. Additionally, we will not make any changes to the study without EC and IRB approval, except where necessary to eliminate apparent immediate hazards to study participants.

We agree to ensure that all staff members involved in the conduct of this study are informed about their obligations in meeting the above commitments.

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Executive Secretary: *Natalya Chalyk PhD*, Institute of Cardiology, 12 Chernyshevskogo Str, 410028 Saratov, Russia

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4. KEY ROLES

Principal Investigator:

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Principal Investigator is ultimately responsible for all aspects of the project including:

A. It is the responsibility of the PI to ensure that each subject is adequately informed and freely consents to participate in the research. The PI will personally assure that every reasonable precaution is taken to reduce risks to the study participants, including subjects who are enrolled initially but prove to be screening failures or for whom data is incomplete, unusable, and/or otherwise eliminated during analysis.

B. It is the responsibility of the PI to delegate responsibility to the research staff in a manner that is commensurate with the staff's training and qualifications.

C. It is the responsibility of the PI to ensure that all procedures associated with the research are performed in accordance with the protocol, including the schedule of events as applicable, with the appropriate level of supervision, only by individuals who are licensed or otherwise qualified to perform them under applicable laws, regulations, and policies.

D. The PI will ensure adherence to the study protocol and monitor the informed consent process.

E. The PI will ensure that there are appropriate facilities and resources to conduct the research.

F. It is the responsibility of the PI to be available to the research staff as needed and to regularly review research processes and address any deficiencies identified through quality improvement processes. The PI may also contact the funding partner to schedule an external assessment of his or her research

through an on-site visit and a regulatory audit of the protocol file. The PI is responsible for maintaining documentation of any quality improvement process for their specific studies.

G. It is the responsibility of the PI to ensure that adequate staff, resources, professional practices and standards of care are maintained at external performance sites.

H. It is the responsibility of the PI to evaluate the intermediate and final results of the project and to take a leading role in the preparation of the final report.

Protocol Chair/Co---Chair:

Protocol Chair --- Yuriy K Bashmakov MD, PhD, yuriy.bashmakov@lycotec.com, Lycotec Ltd, McClintock Building, Granta Park, Cambridge, CB21 6GP, United Kingdom. Tel (44) -1223-42-721 Fax (44)-1223-42-72
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Protocol Chair and Co-Chair are ultimately responsible for all aspects of the study protocol and its implementation including:

A. Writing the protocol document.

B. Ensuring that the necessary approvals are obtained, including those of the review boards, the sponsor, and any others for the protocol and subsequent amendments.

C. Monitoring the study during its execution, which includes: reviewing each case record to confirm eligibility, reviewing each case record to determine compliance with the protocol, reporting adverse events, determining any necessary changes to the protocol and informed consent documents and submitting them as protocol amendments to the clinical site.

Medical Officer --- Victor A Klochkov MD, PhD*, v.klochkov@yahoo.com.*
Institute of Cardiology, 12 Chernyshevskogo Str, 410028 Saratov, Russia

The Medical Officer is ultimately responsible for all aspects of the medical services relating to the study protocol and to the volunteers enrolled in the study including:

A. Provision of medical advice and guidance on the writing, review, approval, and development of the study protocol.
B. Serving as a consultant to other study participants on issues related to the health of individual volunteers enrolled in the study.
C. Leading the analysis and evaluation of medical results from the study.
D. Evaluating complex issues related to the medical safety of the study.

Medical Monitor: Yuriy K Bashmakov MD, PhD yuriy.bashmakov@lycotec.com,
Lycotec Ltd, McClintock Building, Granta Park, Cambridge, CB21 6GP, United Kingdom. Tel (44) -1223-42-721 Fax (44)-1223-42-72

The Medical Monitor is ultimately responsible for all aspects of coordination of work and communications between Lycotec Ltd, UK and Saratov Institute of Cardiology, RF including:

A. Verification of the Investigator's qualifications and resources (clinical base, staff, equipment, laboratory).
B. Verification of Investigator's compliance with the protocol.
C. Control and reporting of findings, processes and conduct of the study.
D. Discussion and negotiation of any adjustments to the study that may become necessary over the course of the study.

Executive Secretary: Natalia Chalyk PhD, Institute of Cardiology, 12 Chernyshevskogo Str, 410028 Saratov,

The Executive Secretary is ultimately responsible for all aspects relating to the documentation of the study including:

A. Preparation and submission of the study protocol to the Institutional Ethics Review Committee according to the timeline defined by the PI.
B. Preparation and uploading of the files for study registration to the International Trial Registry.
C. Communications with all parties involved in preparing amendments to the study protocol should any occur over the course of the study.
D. Proper maintenance of the documentation relating to all stages of the study including intermediate and final reports.
E. Maintaining the timeline for preparation of intermediate and final study reports.

5. LIST OF ABBREVIATIONS

6. PROTOCOL SUMMARY

Full Title: **Systemic Effect of Lycosome-Formulated Dark Chocolate and GA Lycopene on Gut Microbiota, Liver Metabolism, Blood Markers of Oxidative Status and Skin Parameters in Healthy Overweight Volunteers**

Short Title: **Effect of Lycosome-Formulated Dark Chocolate and Lycopene on Gut Microbiota, Liver, Blood and Skin**

Sample Target Size: 30

Study Population: Healthy Caucasian males and females with no acute or chronic diseases aged from 50 to 70 years with body mass index more than 30 and less than 35 kg/m^2.

Participating Sites:

1. Lycotec Ltd, McClintock Building, Granta Park, Cambridge, CB21 6GP, United Kingdom. Tel (44) -1223-42-721 Fax (44)-1223-42-72
2. Institute of Cardiology, 12 Chernyshevskogo Str, 410028 Saratov, Russia

Study Design:

The study is designed as a single, interventional, randomized clinical study to determine if one month's consumption of lycosome-formulated dark chocolate (DC) containing lycopene affects the spectrum of intestinal microbiota, blood lipids, liver parameters and oxidative status of healthy volunteers. The effects of lycopene alone and DC without lycopene addition are in the scope of the control groups as secondary objectives. All volunteers will be asked to give written consent to participation in the study and will be informed about the major goal of the study and its objectives.

The major research question to be answered in the study is whether ingestion of lycosome formulated DC or its two major constituents – lycopene and cocoa flavanols for 1 month can affect the composition of the gut microbiota, liver metabolism, blood markers of biological oxidation and skin parameters in heathy middle-aged overweight individuals.

Therefore, the primary endpoint of the project will be parameters reflecting composition of gut microbiota, blood lipids and markers of serum levels of biological oxidation – malonic dialdehyde, oxidized LDL and certain other values. The secondary endpoints will be skin parameters – sebum, corneocytes and bacterial load. Each of these parameters will be measured before trial initiation ("0" time point) and after 1 month (final point of clinical study).

The study will be conducted following enrolment of 30 volunteers with equal representation in the study population of males and females. The study is planned to include healthy overweight individuals in the age range of 50 to 70 years old, with confirmed health status and no indication of chronic or acute disease. In particular, volunteers with history or immediate signs of

liver disease, alcoholism or intake of any medication (non-steroidal anti-inflammatory drugs, hormones, vitamins and food supplements or energy drinks) will be excluded from participation in the study. Among other exclusion criteria will be pregnancy, extensive tobacco smoking (>10 cigarettes per day), and history of cardiovascular disease. To minimize inconsistencies originating from body weight, patients with Body Mass Index (BMI) from 30 to 35 will be enrolled in the study. All volunteers will be requested to withdraw from consumption of dark chocolate, cocoa and lycopene containing products (tomatoes, ketchup etc.) for 2 weeks prior to study initiation and will be requested to ingest cocoa and lycopene-containing products strictly as mandated by the study protocol.

All study participants will undergo a preliminary anamnestic and clinical investigation. This will include reading the vital signs, determination of body mass index, anthropometry, blood pressure measurement, and electrocardiography. Among the laboratory tests will be determination of fasting serum glucose and lipids (total cholesterol, LDL cholesterol, HDL and total triglyceride levels), determination of ALT and AST level, total bilirubin, and CRP. All volunteers will be also be subject to a standard hematological investigation.

The volunteers will be randomized according to age, gender, body weight and BMI using a simple randomization method and block randomization.

The interventional procedure will be conducted stringently according to the conditions described in the protocol approved by the Institutional Review Board. In particular, volunteers will be given the study products over two separate visits for a total of one month's supply. To assess effect of lycosome-formulated DC volunteers from the first arm will be given lycosome-formulated DC containing 7mg lycopene while volunteers from the fifth arm will be given the regular formulation of DC without lycopene. Volunteers from the second arm of the study will be given capsules containing 7 mg of lycopene, this amount is equal to the amount of lycopene ingested daily with lycosome-formulated DC. Investigational products will be given to volunteers twice – at the "0" time point of the study and at the intermediate time point (two weeks after study initiation). To assess dose-dependency and effect of fat matrix volunteers from the third and fourth arms will be given 30 mg of lycopene in either cocoa butter or sunflower oil.

Upon arrival at the study site all volunteers will be asked about current health status and will undergo scrupulous anamnestic, physical and laboratory investigations. Each of these procedures will be conducted by

highly trained medical professionals including nurses and practicing physicians.

Study products will be given immediately after all evaluations are complete.

Lycosome formulation of DC is a proprietary formulation of DC (Lycotec Ltd, Cambridge, UK) with enhanced bioavailability of lycopene and cocoa flavanols in which the flavanols are protected from oxidation by a lycopene coat and embedded into cocoa butter micelles of the chocolate matrix preserving thereby cocoa flavanols from degradation and oxidation. The final concentration of lycopene in 10 grams of the composite lycosome formulation of DC is 7 mg. The lycopene is in the form of tomato oleoresin from Lycored Inc. (NJ, USA) and contains 97% all trans-isomers and 3% all cis-isomers.

Volunteers will be asked to consume the investigational products in the evening with the main meal once a day for 1 month.

Blood serum specimens will be collected, processed, aliquoted and frozen at
– 80˚C for later analysis at both the “0” time point of the study and after 1 month of the study. All aliquots will be coded without the name of the volunteer being indicated. The identity of specimens will be known only to the Principal Investigator and the Medical Monitor.

Fecal specimens will be collected at the same time points by volunteers in the comfort of their homes and will be required to be delivered to the study laboratory within 1 hour of collection. All fecal specimens will be frozen and stored at the study site until analysed. Should any shipping be required the study specimens will be shipped on dry ice by overnight courier.

Among the biochemical tests conducted on serum specimens will be the activity of liver specific enzymes (alanine aminotransferase and aspartate aminotransferase as well as activity of gamma-glutamyltransferase), level of bilirubin, concentrations of triglycerides, LDL, HDL, total cholesterol and levels of peroxidation products (IOD and LDL-Px).

Biochemical assays will be performed by qualified and licensed personnel using a BioSystems Analyzer and compatible commercial kits of reagents. Standards will be purchased from Sigma-Aldrich only.

Skin swab parameters will assessed on sebum quantity and viscosity, rate of corneocyte desquamation and damage, and total bacterial load of gram-negative bacteria.

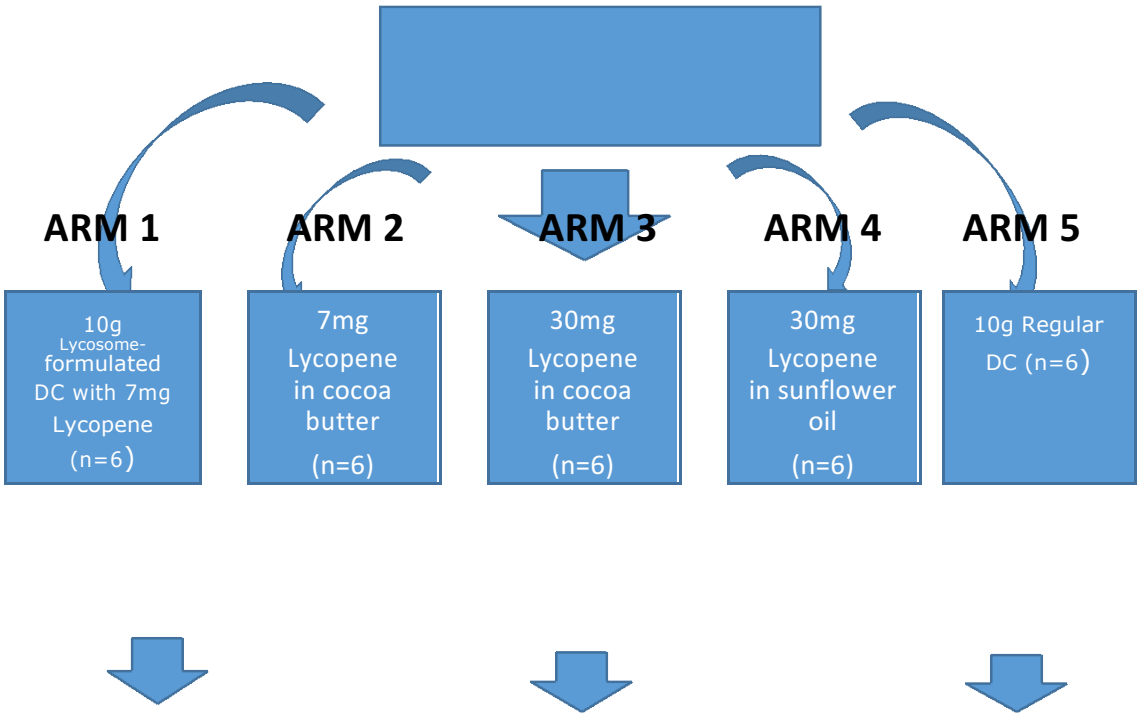
Encoding of the results will performed in collaboration with the Principal Investigator and the Medical Monitor.

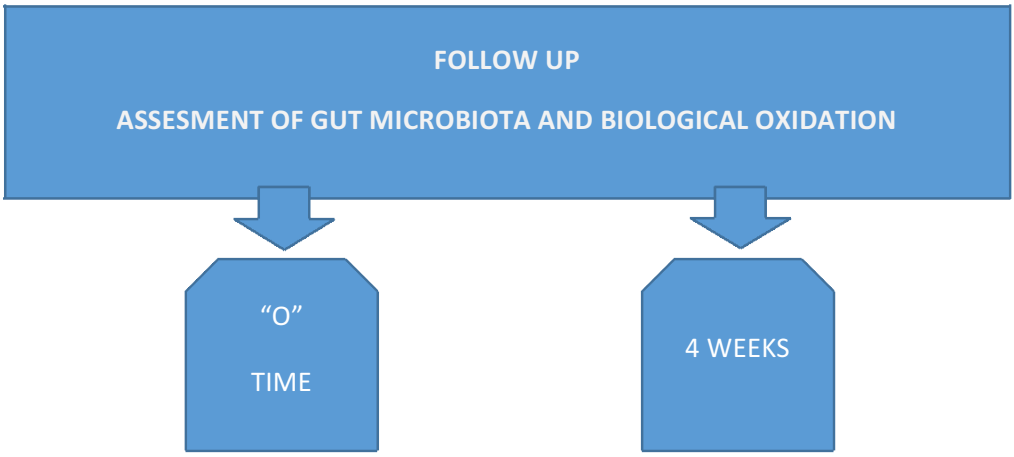
Statistical analysis will performed by an independent statistician using commercially available "BioStat-3" software. The statistician will not be informed of study group names or the nature of interventions in each group.

Packaging and labeling of study products and biological specimens will be carried out according to the guidelines of the NIH and US Department of Health and Human Services, as recommended for biologicals and investigational health food products. The study products will be shipped to the study site in specially designed containers with primary and secondary components. A container closure system will be used to avoid contact of investigational products with light, moisture, excess temperature and air. This packaging system guarantees stability of the products for the intended use. Protection from microbial contamination is provided by maintaining container integrity after the packaging system has been sealed. An adequate and validated procedure will be used for investigational product manufacture and packaging.

All individual packages of lycosome-formulated DC and regular DC as well as lycopene capsules will be labeled with a numerical code. None of the volunteers or study personnel will be informed about coding assignment for the duration of the study.

Study Scheme





Upon arrival at the study site the investigational products will be inspected by the Medical Officer and stored in a locked room at the study site. Only the Medical Officer and Principal Investigator will have access to the investigational products during the study.

Results collected for each volunteer will be stored as both paper files and digital files in Excel format and will be available for access by the Medical Officer and Principal Investigator. To avoid bias each volunteer will be given a random computer code containing numerical characters and three letters.

7. Clinical Protocol

7.1 Introduction:

7.1.1 Background:

The human gastrointestinal (GI) tract contains a complex and dynamic population of microorganisms widely defined as the gut microbiota, which exert a notable influence on the host in health and disease (1). Multiple factors contribute to the establishment and diversity of the human gut microbiota. Diet is considered as one of the main factors

controlling and shaping the gut microbiota throughout life. Intestinal bacteria play a crucial role in maintaining immune and metabolic homeostasis and protecting against pathogens and toxic products of intermediate metabolism, including free radicals (2). Altered gut bacterial composition (dysbiosis) has been associated with the pathogenesis of many inflammatory diseases, infections and possibly biological oxidation (3). The interpretation of these studies relies on a better understanding of inter-individual variations, heterogeneity of bacterial communities along and across the GI tract, functional redundancy and the need to distinguish cause from effect in states of dysbiosis. Our current understanding of the development and composition of the human GI microbiota is that it is highly affected by nutritional factors and impacts the integrity of metabolic pathways in extra-intestinal tissues throughout the body as well as the processes of biological oxidation.

The human GI tract is the largest interface structure (250–400 m^2) in the human body and protects the host from foreign substances and organisms including bacteria, viruses, environmental factors and antigens (4). Over the human lifespan around 60,000 kg of food passes through the human GI tract, along with an abundance of microorganisms from the environment which impose an enormous challenge to gut structure and function (4, 5). The variety of intestinal bacteria in the GI tract termed the ‘gut microbiota’ helps prevent damage and has co-evolved with the host throughout evolution to form an intricate and mutually beneficial relationship (3, 6). The number of microorganisms inhabiting the GI tract has been estimated to exceed 10^{14} which represents about 10 times more bacterial cells

than the number of human cells and over 100 times the amount of genomic content (microbiome) as the human genome (1, 7). Carbohydrate fermentation is one of the main activities of the human gut microbiota, driving the energy and carbon turnover of the colon. Dominant and prevalent species of gut bacteria, including short-chain fatty acid (SCFA) producers, appear to play a critical role in initial degradation of complex plant-derived polysaccharides (8). Other gut bacteria are known to specialize in oligosaccharide fermentation, in particular Bifidobacteria. Supplementary species are known to participate in reductive acetogenesis, reduction of sulfate and methanogenesis (8, 9). Efficient conversion of complex indigestible dietary carbohydrates into SCFA is essential for the nutritional requirements of gut microbial populations as well as for colonic cells which use SCFA for energy requirements (10). Moreover, butyrate and propionate produced by the gut microbiota are essential for intestinal physiology, immune function and possibly mitochondrial respiration, while acetate acts as a substrate for lipogenesis and gluconeogenesis in eukaryotic cells (11). SCFA have also been shown recently to be involved in regulation of immune function, all stages of inflammation and adipose tissue function (12). A wide spectrum of inflammatory mediators regulated by gut microbiota function are essential for adipose tissue function in obesity and type 2 diabetes mellitus (13). The proximal colon is a major site of carbohydrate conversion by the gut microbiota (14). Depletion of carbohydrate content in the distal colon promotes switching of digestive conversion to other substrates, in particular proteins and fat remnants. Their catabolites - ammonia, phenols, p-cresol, certain amines and hydrogen sulfide are highly toxic substances whose accumulation in the colonic lumen may provoke inflammation and cancer (15). Therefore, function

of the gut microbiota is mediated mainly by chemical messengers and metabolites whose production is highly dependent on diet and food constituents.

7.2 Rationale:

Diet and other environmental factors are essential in modulating the composition and metabolic activity of the human gut microbiota, which in turn has a serious impact on health. In particular, macronutrients have a major role in shaping the composition and activity of these complex populations of gut microbiota. It is widely accepted that carbohydrates play the most important role in regulating the microbiota spectrum. The impact of dietary fats on the gut microbiota is less well defined. Western-style diets, rich in fat and proteins, are associated with gut microbial populations that are typified by a *Bacteroides* enterotype whereas traditional diets rich in plant polysaccharides and carbohydrates are associated with a *Prevotella* enterotype. Vegetarian dietary patterns are more likely associated with an abundance of *Clostridium* clusters (16).

It is suspected that dietary polyphenols sourced from many foods including grapes, grains, tea, cocoa and berries, may have some impact on gut microbiota (17). However, there are no systematic studies devoted to this question. Similarly, the effect of DC on the gut microbiota remains largely unknown. It is completely unknown for now whether lycopene, a major carotenoid from tomatoes, may have any effect on intestinal bacteria. Moreover, the relationship between the intestinal microbiota and biological oxidation systems has never been investigated.

It has to be emphasized that use of cocoa-derived polyphenols and lycopene in clinical studies is a very challenging task. Each of them has

a low bioavailability rate due to the hydrophobic nature of their amphiphilic molecules. Therefore, clinical studies aimed at the investigation of biological functions of lycopene and cocoa flavanols may require special nutraceutical formulations of these compounds with enhanced bioavailability. A newly developed lycosome formulation of DC (Lycotec Ltd, Cambridge, UK) contains both lycopene and cocoa flavanols in highly bioavailable forms (18). This formulation was developed by implementation of different microencapsulation techniques (spray drying, ultrasound, supercritical CO2) which allows incorporation of bioactive compounds (lycopene and cocoa flavanols in this particular case) into the structure of lycosomes which are particles with an increased resistance to the acidic environment of the stomach and higher ability to be absorbed in the intestine (19). Besides the compounds of interest (lycopene and cocoa flavanols), lycosome particles (coco-somes) contain a phospatidylcholine (used as a hydrophilizing and emulsifying agent).

Moreover, the lycopene used in lycosome-formulated DC is a well known antioxidant whose anti-radical capacities exceed the anti-radical capabilities of all other carotenoids multifold (20). Therefore, ingestion of lycosome-formulated DC may translate into significant attenuation of free radical formation in the human body. This creates the rationale to investigate parameters of biological oxidation in volunteers ingesting lycosome-formulated DC.

Altogether the factors mentioned above support the main objective of our study, creating a rationale for trying to investigate the effect of lycosome-formulated DC on microbiota spectrum and oxidation processes in healthy volunteers.

Each of these questions will be addressed in the current study.

7.3 Clinical Study Objectives:

7.3.1 Primary objective:

The primary objective of this clinical study is to verify the effect of lycosome-formulated dark chocolate (DC) and its two major ingredients – lycopene and cocoa flavanols on the spectrum of gut microbiota in healthy middle–aged overweight volunteers.

Secondary objective:

To investigate the effect of lycosome formulated dark chocolate (DC) and its two major ingredients – lycopene and cocoa flavanols on liver metabolism, blood lipids, marker of oxidative and inflammatory damage, and skin parameters in healthy middle-aged overweight volunteers.

7.4 Study Design

7.4.1 Selection and randomization of volunteers

Medical personnel at the outpatient clinic of Saratov Institute of Cardiology will be informed about implementing the study, its major goal and selection criteria for volunteers. Suitable individuals will be invited for preliminary check-up (physical and laboratory investigation) during the initial phase of enrolment. All suitable individuals will be re-screened after the wash-out period of the study before the final decision on study enrolment is made.

Randomization of volunteers in the study will be performed using widely accepted methods such as simple randomization and stratified randomization. Briefly, the

software containing a random number generator will be applied to the database of volunteers. The assigned group will be considered as final. The groups will be balanced according to numerical age and gender with special software. Stratified randomization will be used to enhance statistical strength of the final results and will ensure equality of groups for secondary selection criteria.

7.4.2 Schedule of Procedures/Evaluations

All medical evaluations will be performed in a window of time from 0-15 days of study initiation. This includes anamnestic evaluation with preliminary physical and laboratory investigation. All volunteers will be re-screened at the "0" time point of the study.

7.4.3 Study Duration

Interventional period is designed to last for 1 month. It will be preceded by the enrolment period.

Inclusion Criteria

Major inclusion criteria are as follows: Caucasian male or female subjects 50-70 years old, overweight with BMI more than 30 and less than 35 kg/m^2, absence of concomitant intake of anti-hypertensive, lipid-lowering or

any other cardio-vascular drugs, vitamin supplements and any specific dietary interventions.

7.4.4 Exclusion Criteria

Among exclusion criteria are: inability to comply with the study protocol, severe medical conditions (hepatitis, pancreatitis, uncontrolled diabetes, cancer, recent cardiovascular events, tuberculosis etc.), history of alcohol abuse or alcoholism, participation in other clinical trials and intolerance of cocoa-based or lycopene-containing products.

7.4.5 Wash-out period

The volunteers will be asked to refrain from consumption of cocoa and tomato based products for 14 days before beginning the study.

7.4.6 Mode of intake

The volunteers will be asked to take the study products, one 10 gram bar of lycosome formulated DC, 1st arm, or one 10 gram bar of regular DC, containing no lycopene, 5th arm, once a day in the evening with the main meal for 1 month.

Volunteers from the 2nd arm of the study will be requested to take a capsule containing 7 mg of lycopene, an amount equal to the quantity of lycopene in the 10 g bar of lycosome formulated DC.

Volunteers in the 3rd arm will be asked to take capsules with 30 mg lycopene in cocoa butter, and in the 4th arm 30 mg of lycopene in sunflower oil.

All interventional products are to be ingested once a day in the evening with the main meal for 1 month.

8. STUDY DRUG STORAGE AND ACCOUNTABILITY

The Principal Investigator and Medical Officer of the study are responsible for ordering, receiving, and tracking inventory; storing,

dispensing, and returning study products properly; and, where necessary, labeling of study products prior to dispensing according to protocol guidelines and good manufacturing practices (GMPs). All study products supplied for the protocol will be accounted for and tracked in a manual or electronic accountability log for the study. Accountability for the study products will be documented from the time of initial receipt through dispensation and final disposal of leftover study products. The accountability log will indicate the date, amounts, batch numbers, and condition upon receipt of all materials received. Upon study completion, remaining study products will be returned or destroyed as defined by specific Principal Investigator instructions, or other documented instructions. The balance returned or sent for destruction will be recorded in the study accountability log. Quality assurance reviews and inspection of study products and accountability documentation will be performed at intervals during the study by a Clinical Monitor. Study products will be stored at the clinical site facility in a locked cabinet.

9. STUDY PRODUCT COMPLIANCE AND ADHERANCE

Compliance of volunteers to the protocol and adherence to the wash-out period will be determined by questioning the volunteers during the intermediate and final visits to the clinical site and by random private phone calls. Volunteers with poor adherence to the study protocol will be withdrawn from the study by the Medical Officer.

10. Concomitant Medications

Patients with concomitant intake of anti-hypertensive, lipid-lowering or any other cardio-vascular drugs are not eligible for the study.

11. Safety and Effectiveness Assessments:

Safety assessment procedures are barely applicable since widely consumed food products will be used for the study. Volunteers with DC and tomato product intolerance will not be enrolled for the study.

Effectiveness assessment will be by biochemical and bacteriological analysis following ingestion of study products.

11.1 Safety assessments

The volunteers will be instructed to look out for the appearance of skin rash, nausea or dyspepsia and to report any of these to the Medical Officer should they occur.

11.2 Effectiveness assessments

Measurements of gut microbiota spectrum and markers of oxidation in blood will be used for effectiveness assessment.

12. Adverse Event Reporting:

Occurrence of adverse events in volunteers consuming lycopene or DC is very unlikely. Should any occur it is the responsibility of the Medical Officer and the Principal Investigator to determine and analyze adverse reaction cause.

Adverse reactions following lycopene formulated DC product intake can include skin rashes, dyspepsia, transient increase of blood pressure, abnormalities in sleep.

Occurrence of any of these events will be considered as a reason for volunteer withdrawal from trial participation.

12.1 Recording/Reporting requirements

Each of the volunteers will be required to report any possible adverse effect to the Medical Officer assigned to the study, whose responsibility is to immediately inform the Principal Investigator about any possible adverse reaction. Moreover, appropriate information will be immediately passed on to the Local Ethical Committee. All observed or volunteered adverse events

(serious or non-serious) and abnormal test findings, regardless of study group or suspected causal relationship to the study will be recorded in the subject case histories. Adverse events or abnormal test findings felt to be associated with the study will be followed up until the event (or its sequelae) or the abnormal test finding resolves or stabilizes.

For all adverse events, sufficient information will be pursued and/or obtained so as to permit 1) an adequate determination of the outcome of the event (i.e., whether the event should be classified as a *serious adverse event*) and; 2) an assessment of the casual relationship between the adverse event and the study products.

An abnormal test finding will be classified as an *adverse event* if one or more of the following criteria are met:

- The test finding is accompanied by clinical symptoms

- The test finding necessitates additional diagnostic evaluation(s) or medical/surgical intervention; including significant additional concomitant drug treatment or other therapy

Lycotec Ltd and the Saratov Institute of Cardiology will promptly review documented adverse events and abnormal test findings to determine 1) if the abnormal test finding should be classified as an adverse event; 2) if there is a reasonable possibility that the adverse event was caused by the study products; and 3) if the adverse event meets the criteria for a *serious adverse event*.

If the final determination of causality is “unknown and of questionable relationship to the study product”, the adverse event will be classified as *associated with the use of the study product* for reporting purposes. If the Sponsor-Investigator’s final determination of causality is “unknown but not related to the study product”, this determination and the rationale for the determination will be documented in the respective subject’s case history.

12.2 Withdrawal of subjects due to adverse events

Any subject with adverse reaction and/or abnormal laboratory test findings will be withdrawn from the study by the Medical Officer and replaced with an eligible volunteer from the pre-screened pool of volunteers. An appropriate report to the Local Ethical Committee will be filed.

13.Statistical Methods/Data Analysis: 9.1

Study endpoint

For assessment of normally distributed parameters the Shapiro-Wilk method will be used. Student's t-test will be applied both for paired and unpaired samples. Between-group differences at one time point will be evaluated by Wilcoxon-Mann-Whitney test (continuous variables) and Fisher's Exact test (categorical variables). Data analysis will performed using Stata SE, version 12.1. All statistical tests are two-sided and statistical significance level alpha will be set at 0.05 for the analysis.

9.2 Sample size determination

Sample size was determined empirically with a preliminary pilot clinical study.

10.1 Statistical effectiveness analysis

Statistical analysis of results will be performed by an independent statistician. If the statistical methods as well as the statistical settings listed above are not appropriate for newly obtained results due to specificity of variant distribution, other methods of statistical analysis will be applied. Any deviations from the statistical plan described above will be explained and justified in a protocol amendment and/or in the final report submitted to the Institutional Ethics Committee.

11.1 QUALITY CONTROL AND QUALITY ASSURANCE

Independent monitoring of the clinical study for protocol and GCP compliance will be conducted periodically by Institutional Ethics Committee qualified staff from Saratov Institute of Cardiology.

Saratov Institute of Cardiology will grant direct access for the study monitors and appropriate regulatory authorities to the study data and to the corresponding source data and documents to verify the accuracy of this data.

12. DATA HANDLING AND RECORDS

12.1 Data recording/Case Report Forms

A Case Report Form (CRF) will be completed for each subject enrolled into the clinical study. The Sponsor-Investigator will review, approve and sign/date each completed CRF; the Sponsor-Investigator's signature serving as attestation of the Sponsor-Investigator's responsibility for ensuring that all clinical and laboratory data entered on the CRF are complete, accurate and authentic.

Source Data are the clinical findings and observations, laboratory and test data, and other information contained in Source Documents. Source Documents are the original records (and certified copies of original records); including, but not limited to, hospital medical records, physician or office charts, physician or nursing notes, subject diaries or evaluation checklists, dispensing records, recorded data from automated instruments, etc. Where applicable, information recorded on the CRF shall match the Source Data recorded on the Source Documents.

12.2 Record maintenance and retention

The Sponsor-Investigator will maintain records in accordance with Good Clinical Practice guidelines; to include:

- Financial disclosure information (i.e., for the Sponsor-Investigator and for sub-investigators who will be involved in the administration of the study products and/or the evaluation of research subjects [i.e., who will contribute significantly to the research study data])
- Curriculum vitae for all participants (i.e., for the Sponsor-Investigator)
- Certificates of required training; e.g., human subject protection, Good Clinical Practice, etc. (i.e., for the Sponsor-Investigator and for all sub-investigators who will be involved in the administration of the study products and/or the evaluation of research subjects [i.e., who will contribute significantly to the study data])
- Listing of printed names/signatures. (i.e., for the Sponsor-Investigator and for all sub-investigators who will be involved in the administration of the study products and/or the evaluation of research subjects [i.e., who will contribute significantly to the study data])
- Normal value(s)/range(s) for medical/laboratory/technical procedures or tests included in the clinical protocol
- Laboratory certification information
- Instructions for on-site preparation and handling of the investigational products and other study-related materials (i.e., if not addressed in the clinical protocol)
- Responsibility delegation log
- Signed informed consent forms
- Completed Case Report Forms; signed and dated by Sponsor-Investigator
- Source Documents or certified copies of Source Documents
- Monitoring visit reports
- Copies of Sponsor-Investigator correspondence (including notifications of safety information) to sub-investigators
- Subject screening and enrolment logs
- Subject identification code list

• Investigational product accountability records, including documentation of disposal.

- Final clinical study report
- Decoding procedures for blinded trials
- Master randomization list
- Retained biological specimen log
- Interim data analysis report(s)

The Sponsor-Investigator will retain the specified records and reports for up to 5 years.

13. ETHICS

13.1 Institutional Review Board (IRB) approval

The Sponsor-Investigator will obtain from the Saratov Institute of Cardiology Institutional Review Board (IRB) and Ethics Committee, prospective approval of the clinical protocol and corresponding informed consent form(s); modifications to the clinical protocol and corresponding informed consent forms, and advertisements (i.e., advertisements directed at potential research volunteers) for study recruitment.

13.2 Ethical and scientific conduct of the clinical research study

The clinical research study will be conducted in accordance with the current IRB-approved clinical protocol; ICH GCP Guidelines adopted by the FDA; and relevant policies, requirements, and regulations of Saratov Institute of Cardiology and applicable regulations of the United Kingdom and the Russian Federation Health authorities.

13.3 Subject informed consent

The Sponsor-Investigator will make certain that an appropriate informed consent process is in place to ensure that potential research subjects, or their authorized representatives, are fully informed about the nature and objectives of the clinical study, the potential risks and benefits of study participation, and their rights as research subjects. The Sponsor-Investigator, or sub-investigator(s) designated by the Sponsor-Investigator, will obtain the written, signed informed consent of each subject, or the subject's authorized representative, prior to performing any study-specific procedures on the subject. The date and time that the subject, or the subject's authorized representative, signs the informed consent form and a narrative of the issues discussed during the informed consent process will be documented in the subject's case history. The Sponsor-Investigator will retain the original copy of the signed informed consent form, and a copy will be provided to the subject, or to the subject's authorized representative.

The Sponsor-Investigator will make certain that appropriate processes and procedures are in place to ensure that any ongoing questions and concerns of enrolled subjects are adequately addressed and that the subjects are informed of any new information that may affect their decision to continue participation in the clinical study. In the event of substantial changes to the clinical study or the risk-to-benefit ratio of study participation, the Sponsor-Investigator will obtain the informed consent of enrolled subjects for continued participation in the clinical study.

14. STUDY DISCONTINUATION CRITERIA

14.1 Discontinuation of individual research subjects

Individual research subjects can be withdrawn from the study due to occurrence of adverse effects, volunteer's request (regardless of reason) or non-compliance with the protocol. Volunteers withdrawn from the study will be replaced from the pre-screened pool of volunteers.

14.2 Sponsor-Investigator discontinuation of the clinical research study

The clinical study can be terminated by mutual agreement between Sponsor and Investigator for any reason. The Institutional Review Board (IRB) and Saratov Institute of Cardiology Ethics Committee will be notified promptly of discontinuation of the entire clinical study.

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