Report on Short TermScientificMission (STSM) to University of Trento, Centre for Computational and System Biology – COSBI,Rovereto(IT)

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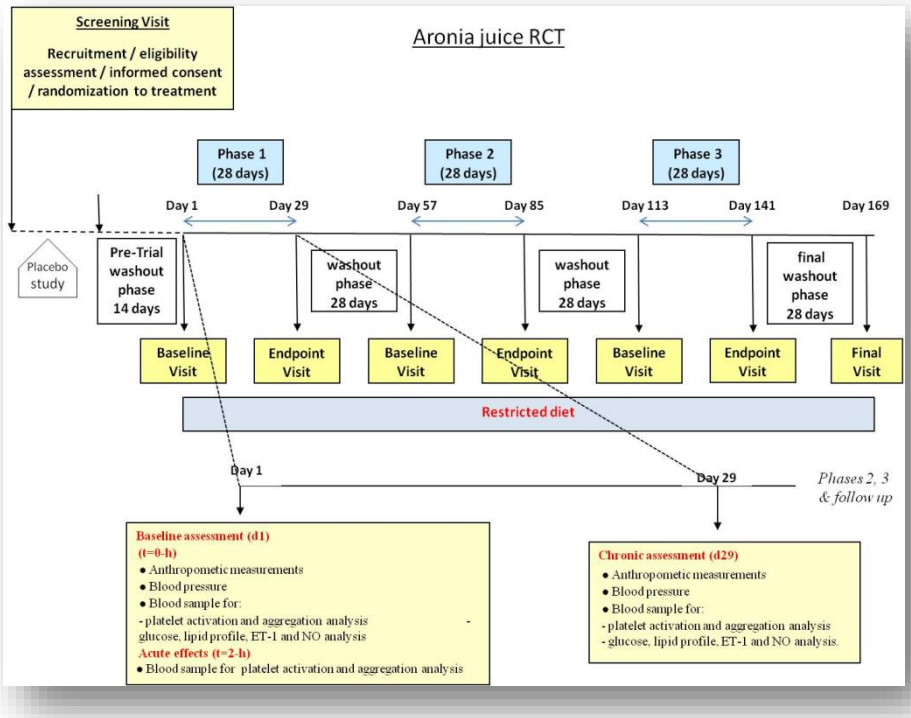
Inter-individual variability in response to plant bioactive on platelet function – DATA ANALYSIS

Introduction:

Understanding, analyzing, interpreting results and visualizing critical relationships across massive amounts of unstructured data including metabolome and metabiome data, dietary profiles, biomarkers, mechanisms of action, final outcomes as suggested determinants and/or variables, towards applicable conclusions is challenging. However it depends on optimal solutions for data mining and the use of advanced specifically developed algorithms.

The main objective of this STSM was to perform advanced data analysis applied on clinical study which investigatesthe effects of polyphenol rich foodon the platelet function.

The study was designed as randomized, cross.-over, three arm, placebo controlled clinical trial, in apparently healthy subjects. It aimed to investigate the effects of aronia juice polyphenols on platelet function.



Study population

- 88 participants enrolled (83 completed), both males and females, aged between 35and 50 years

According to the defined inclusion criteria (apparently health subjects with or without CVD risk factors, a heterogeneous distribution of cardiovascular disease risk factors was observed, with 15 subjects without any of traditional risk factors and 17 subjects with metabolic syndrome, defined as clustering of at least three of five CVD risk factors (obesity, low HDL, triglycerides, high fasting glucose, high blood pressure.

Intervention

Three intervention arms were:

- High polyphenol arm: 100 ml of pure aronia juice per day (appx 1000 mg of total polyphenols)
- Low polyphenol arm: 100 ml of the beverage with 1/4 of the juice and 3/4 of placebo per day (providing 250 mg of total polyphenols)
- Placebo arm: 100 ml of placebo per day (the same nutrient composition and sensory characteristics but without polyphenols).

The duration of each intervention in cross-overdesign was 4 weeks with 4 weeks of wash-out between each intervention.

Dietary restrictions included consumption of aronia berries and related products, other berries and supplements. It was not controlled for polyphenol intake through other foods.Final dataset contained the following information (83 completed participants.

The primary outcomes were:

- two biomarkers of platelet activation (the expression of P-selectin and GPIIbIIIa on platelets (% of P-selectin/GPIIbIIIa positive platelets in the total platelet pool) two biomarkers of heterotypic platelet activation, platelet-monocyte (PMA) and platelet-neutrophil aggregates (PNA), as percentage of aggregates in the total number of monocytes and neutrophyls, respectively

All biomarkers of platelet function were measured by using flow cytometry.

Methods

By applyingthe state-of the art techniques and models for statisticalanalysisweanalysed data from the wholecohort and defined sub-population for 37 variables (for 12 biomarkers of plateletfunction and 25 biochemical and anthropometricbiomarkers). Initially, the statisticalanalysison the effects of the interventionwasperformed on the wholestudycohort, and inthreesubgroups of participantsdefined by clustering CVD riskfactors (group 1. no CVD riskfactors, group 2. with one or tworiskfactors; group 3. with three and more riskfactors). Riskfactorsformetabolicsyndrome are definedaccording to the criteria of the International DiabetesFederation [1] included: waistcircumference ≥ 94 cm for males, ≥ 80 females;systolic/diastolicblood pressure ≥ 130/≥85 mmHg; glucose ≥ 5.6 mmol/l; triglycerides ≥1.7 mmol/l; HDLcholesterol< 1.03 mmol/l for males, < 1.29 mmol/l for females)

Initially, data pre-processing was performed byapplying PCA analysis for the set of baseline clinical dataof ineach of three intervention periods, as a model of quality assessment (the lack of the batch effect between the intervention periods).

Subsequently, alldata weretested for normaldistribution by using the Shapiro-Wilk's test for normality. Log transformationwasappliedon data thatwerenotnormallydistributed. Resultswereconsideredsignificantat $p < 0.05$. Data wereanalyzed for the effects of the interventionon allof 12 parameters of plateletfunction, meanplatelet volume, glucoselevels, differentparameters oflipidstatus (TG, HDL, LDL, and totalcholesterol), body weight, waistcircumference andblood pressure.Willcoxon test and twowaysmixedfactorialAnovawereapplied to test the effect of the long termintervention (baseline vs placebo, baseline vs low dose and baseline vs high dose) on the wholecohort of trial participants.

The sameanalysiswereperformed for the effectsof acute consumption baseline vs placebo/low dose/high dose, for markers of plateletfunction, bloodglucose, blood pressure and TG levels, also by univariateanalysis, takinginto account thatthisissub study of the cross-over RCT, and itisparalleldesigned.

Additionalfactorsthathavebeenconsideredas a confounding for the effect of interventions are BMI (normalweight, overweight, obese), age, sex and polyphenolintakeassessed by 24 hour recall (lowintake: < 600 mg/day, normalintake: 600-750 mg/day and high intake:> 750 mg/day) [2]. TwowaysmixedfactorialAnovawasapplied to test for the effect of intervention for the confoundingfactors.Effect of the intervention, with respect to confoundingfactors,wasanalyzed by applyingtwo way mixedfactorialanova for the foldchange - beforeintervention/afterintervention (intervention*(CVD riskfactor, age, sex, BMI, polyphenolintake).

The betweengroupdifferencewasanalysed by Friedman Anovaas anon-parametric model on absolute and fold (relative) change.

The correlation between values of different biomarkers of platelets activation and the association in the effects of the intervention on biomarkers was performed by Spearman rank correlation.All analyses were performed using R software package [3].

Results

The results of the PCA (principal component analysis) performed on 37 variables are presented as plots in Figure 1. The equally distribution observed have indicated the lack of batch effect between 3 phases of the study. Similarly, PCA plot for the platelets biomarkers presented in Figure 2 confirmed the lack of the batch effect and the suitability of the design (including the defined wash-out period and the validity of the biomarker and method used)

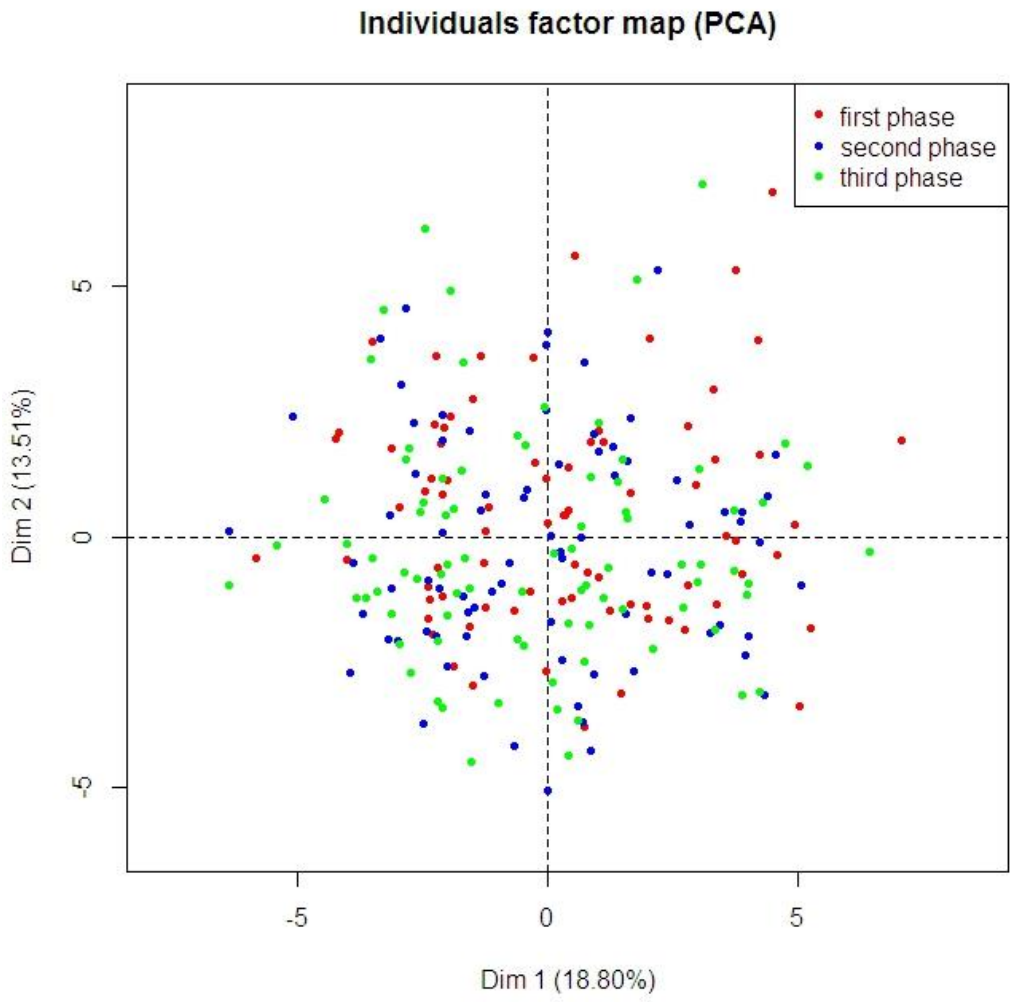


Figure 1*PCA plot – baseline for the three phases (all variables)*

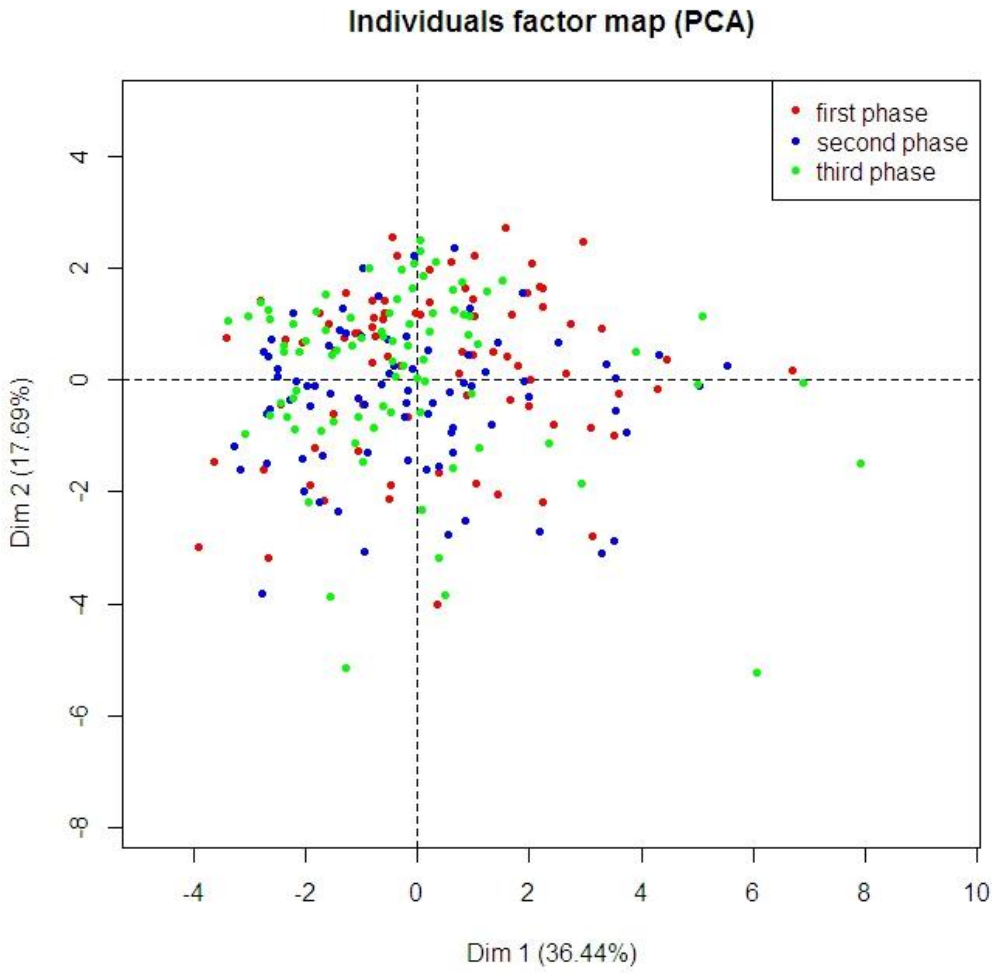


Figure 2*PCA plot – baseline for the three phases (biomarkers of platelet activation)*

Variableswhichreflectbiochemical and anthropometricstatus ofparticipantshavenotbeensignificantlychangedafter the intervention. Theyallvary in the same way withineach of the interventionphases (placebo, low dose and high dose). Significantlychange in twoof twelvebiomarkers of plateletactivation (PMAOp,PNAOp) after the interventionwithbothlow and high dosewasobserved, whileone of them (PMASOp)significantlychangeonlyafter the high dose. Therewere no statisticaldifferences for the samebiomarkersbetweenthe baseline and after the placebo intervention (table1). Box plots in figure 3 show thatlevels of PMAOp,PNAOp andPMASOpwhichalldecreasedafter the interventions with low and high dose of aroniajuicewhichconfirm the hipotesysthataroniahaseffect on biomarkers of plateletactivation.Other biomarkers of platelet activation vary in the same way for both measurements, before and after interventions.

Table 1 Effect of the chronic intervention on the biomarkers of platelet activation

			Placebo		
	before		**after**		
Biomarker	**Median**	**SD**	**Median**	**SD**	**p value (Wilcoxon test)**
PMAOp	51,48	18,99	50,13	18,67	0,84
PMASOp	25,355	18,12	24,6	16,16	0,784
PNAOp	27,945	13,72	26,61	13,04	0,482
			Low dose		
PMAOp	54,74	15,61	48,225	17,08	**0,007**
PMASOp	25,79	13,47	23,78	15,59	0,162
PNAOp	30,1	11,13	26,49	11,9	**0,014**
			High dose		
PMAOp	53,02	18,32	46,35	17,41	**0,019**
PMASOp	25,96	14,88	23,45	13,38	**0,049**
PNAOp	30,23	13,66	24,43	13,02	**0,007**

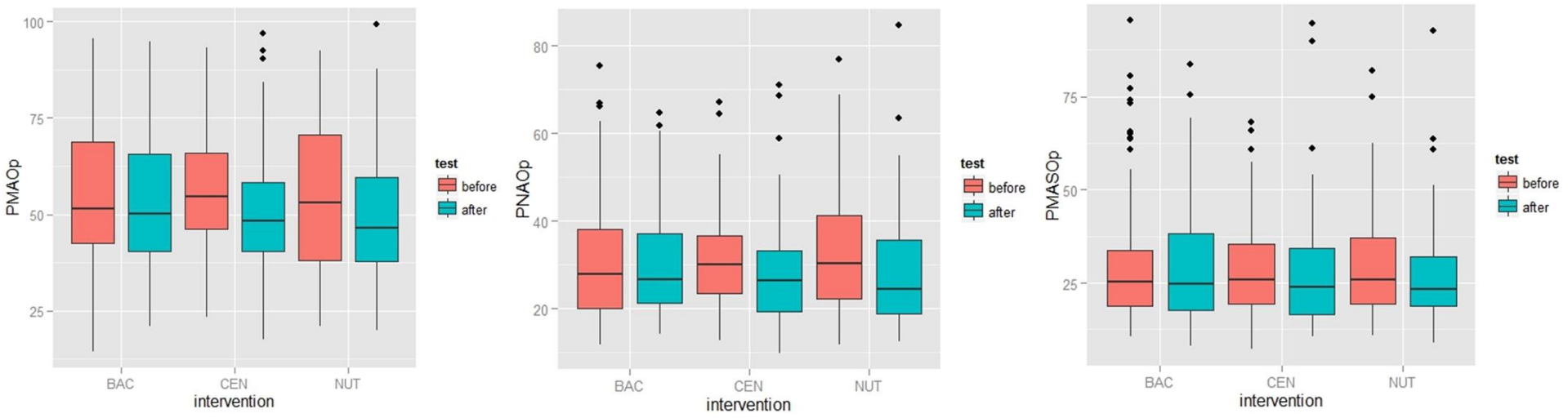


Figure 3*Boxplot diagrams for the biomarkers of platelet activation which are significantly changed after the chronic intervention*

For three out of twelve biomarkers of platelet activation, significant interaction between the intervention and CVD risk factors was shown. PNAOp ($p = 0.018$) and PMASOp ($p < 0.0576$) decreased linearly for the level of intervention but only for the participants without any of the CVD factors. It also decreased among participants with one or two CVD risk factors but the level of decreasing doesn't differ between low and high dose (figure 4 and 6). PNASOp ($p < 0.0492$) figure 5??? Interactions between intervention and other confounding factors were not statistically significant.

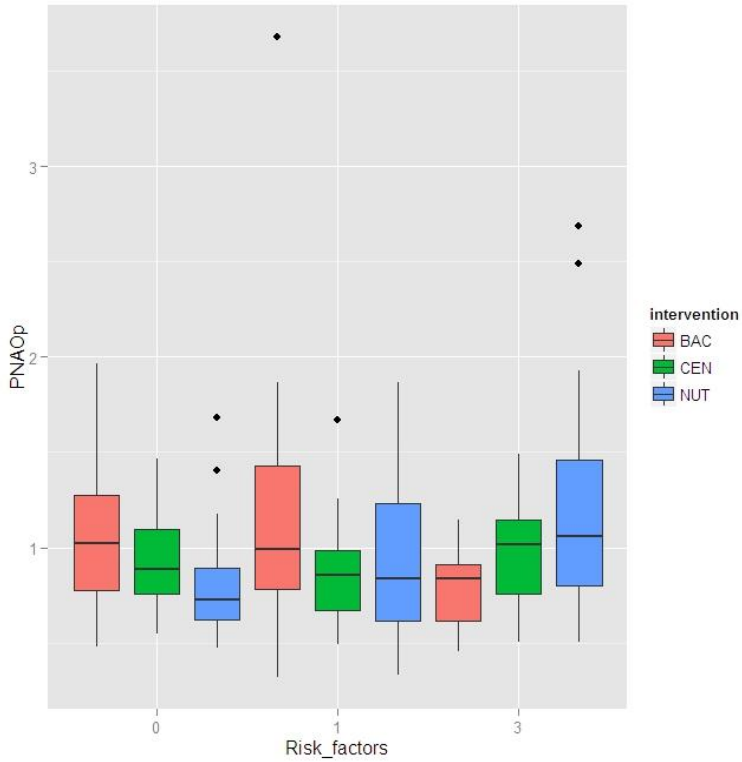


Figure 4 *Box plot diagram for the fold changes for the PNAOp (after/before) for the interaction of intervention and CVD risk factors*

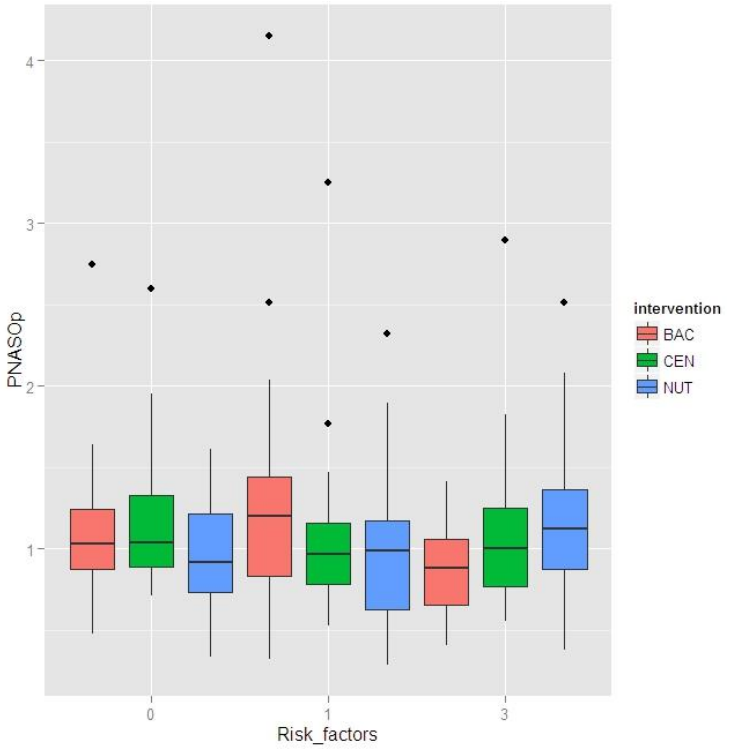


Figure 5 *Fold changes for the PNASOp (after/before) for the interaction of intervention and CVD risk factors*

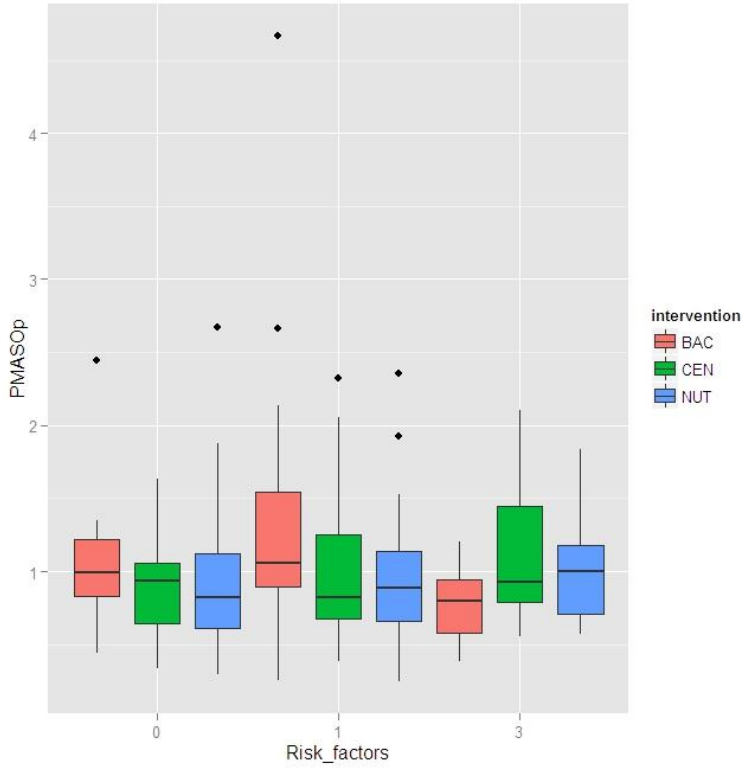


Figure 6*Fold changes for the PMASOp (after/before) for the interaction of intervention and CVD risk factors*

After an acute intervention, two biomarkers were significantly changed.GPIIbIIIaBl decreased after the high dose while PselSOp decreased after both low and high dose (table 2, figure 7.

Table 2*Effect of the acute intervention on the biomarkers of platelet activation*

	BAC				
	before		**after**		
Biomarker	Median	SD	Median	SD	p value
GPIIbIIIaBl	1	0,51	1,06	0,5	0,276
PselSOp	32,32	13,54	31,02	15,29	0,107
			CEN		
GPIIbIIIaBl	0,96	2,22	1,06	0,56	0,456
PselSOp	40	13,81	33,93	13,91	**0,01**
			NUT		
GPIIbIIIaBl	1,15	0,45	0,76	0,36	**0,037**
PselSOp	34,21	15,77	29,31	14,53	**0,038**

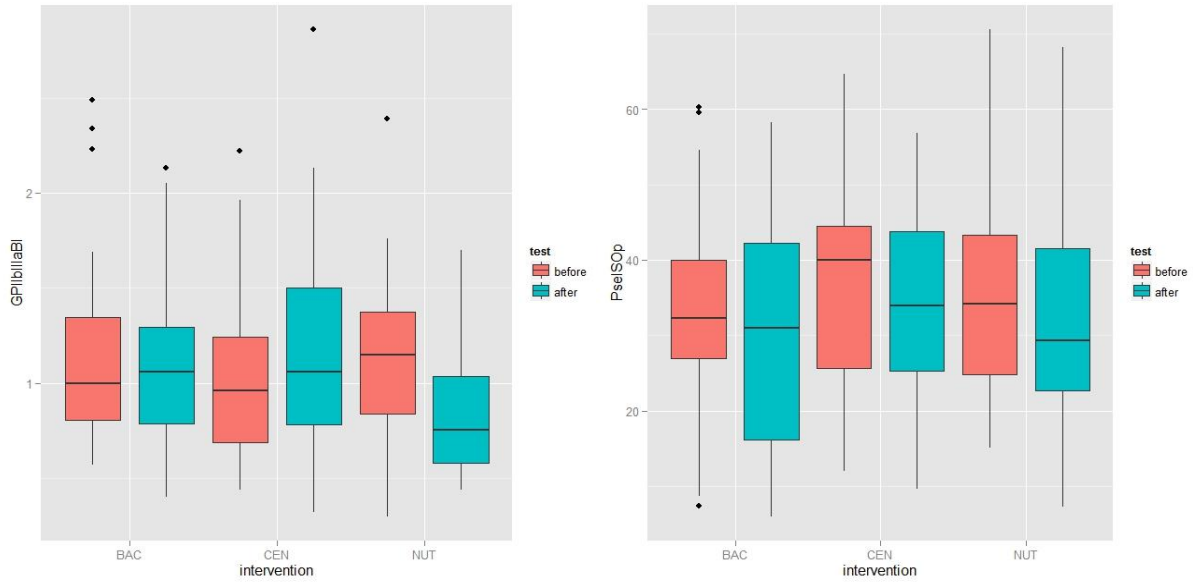


Figure 7*Boxplot diagrams for the biomarkers of platelet activation which are significantly changed after an acute intervention*

Conclusion:

The training in using R language for the assessment of the effect of the cross-over designedstudywasperformed. The use of multiple models of statisticalanalysis (PCA, non-parametric and parametricmodels of univariate and multi-variate analysis) contributed to defining “the best practice” in the evaluation of putative determinants of inter-individualvariation in response. Traditionalriskfactors for CVD identifiedas the maincontributingdeterminantwill be evaluatedindividually, as part of future collaboration. Additionaldeterminants, includinggeneticpolymorphisms, baseline diet and the bioavailability of bioactivecompoundsassumed to be responsible for the observedeffectswill be assessed.

Future collaboration:

As part of STSM, we defined additional work to be performed at the home institution with the support of responsible scientists from the host institution. It included the analysis of the effects of the intervention in subgroups of study cohorts defined by individual risk factors that are known to influence platelet function. We will also access the correlation of the effects with the levels of biomarkers that define those risk factors, analysis of the effects by taking the levels of bioactive metabolites in plasma as determinant of the variability (experimental work has been postponed for the 2016, thus the analysis is going to be performed afterwards); retrospective analysis of the effects in three sub-groups of the study population defined by the single nucleotide polymorphism in gene coding. Futhermore, genetic associations between genetic markers and the response will

be perform using two activation markers of platelets as quantitative trait, P-selectin expression on platelets, and platelet-monocyte aggregation, known to be mediated by P-selectin.
Future collaboration will also include joint work on additional models of statistical analysis (initially not a part of STSM project) based on expertise of both host and home institution, such as network/pathways analysis.to identify functional connections between distinct molecular processes. A metabolic/protein-protein interaction network representing interactions between aronia juice polyphenols and platelet function in humans will be constructed from public molecular interaction databases and by data mining the literature. This network will provide informative preliminary detail on the molecular systems linking polyphenols and platelet function.

References:

[1] K. G. M. M. Alberti, P. Zimmet* and J. Shaw*, Metabolicsyndrome—a new world-wide definition.A Consensus Statement from the InternationalDiabetesFederation,, Journal compilation © 2006 Diabetes UK.*Diabetic Medicine* 23469–480

[2] Anna Tresserra-Rimbau, Eric B Rimm, Alexander Medina-Remón et all,Polyphenolintake and mortalityrisk: a re-analysis of the PREDIMED trial, Tresserra-Rimbauet al. BMC Medicine 2014, 12:77, http://www.biomedcentral.com/1741-7015/12/77

[3]R Development Core Team: R: A language and environment for statisticalcomputing. R Foundation for Statistical Computing, Vienna, Austria. 2005, http://www.r-project.org