Supplementary appendix

This appendix presents details of Promic study

Supplement to: Lifestyle, anthropometric factors and clinically significant prostate cancer diagnosis in men with suspected prostate cancer

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2. Supplementary methods

2.1 Promic study design

Promic (Prostate Metabolism, Cancer Risk and Gut Microbiota, NCT06116851) is a review board approved, single-institution, prospective translational observational cohort trial investigating the interaction between GM and PCa carcinogenesis, as well as the influence of lifestyle, anthropometric factors and germline genetics on GM and PCa risk. The primary objective of the study is to validate our preliminary findings of association between GM and PCa and describe favorable and unfavorable GM composition in regards of prostate cancer risk (20). Exploratory objects include:

1. To investigate the mechanism of how GM affects the prostate carcinogenesis through metabolic changes within the gut, systemic circulation, and prostate tissue.
2. To investigate factors contributing to GM and PCa risk, and potential GM modification methods

Promic study has four (4) cohorts. Cohorts and collected biological samples are summarized in the adjacent table below. For eligibility, subject's detailed medical history is reviewed and documented. General inclusion criteria for all study subjects: 1. Provision of signed and dated informed consent form, 2. Ability and stated willingness to comply with all study procedures and availability for the duration of the study and 3. Male aged > 18 years. The only exclusion criteria include inability to comply with study procedures or unwillingness to participate in the study. Additional inclusion criteria for separate cohorts are listed below:

1. Suspicion cohort (N=298)
    - Clinical suspicion for PCa and indication for PCa diagnostics based on any of the following: 1) elevated PSA (>2.5 ng/ml), 2) palpable prostate

tumor/nodule, 3) image finding suggestive of PCa. Based on the outcome of the PCa diagnostics and planned clinical treatment, subjects may be eligible to other cohorts as well.

2. Cancer cohort
    - RALP patients: clinically significant prostate cancer planned to be treated with Robotic assisted laparoscopic radical prostatectomy (RALP) with or without pelvic lymph node dissection.
3. Benign cohort
    - TURP patients: benign prostate hyperplasia (BPH) planned to be treated with transurethral resection of the prostate (TURP).
4. Control cohort
    - Cystoprostatectomy patients: bladder cancer planned to be treated with removal of the urinary bladder and prostate (cystoprostatectomy).

2.1.1 Study visits and procedures

2.1.1.1 Suspicion cohort

Study visits and procedures for suspicion cohort are presented in Supplementary Figure 1. Patients referred to the Turku University Hospital Department of Urology based on clinical suspicion of PCa were screened for eligibility. At the screening visit, participants who provide written informed consent undergo study procedures, beginning with detailed questionnaires (Supplementary appendix 2.2). Anthropometric measurements (height, weight, BMI, and waist circumference) are recorded by the investigator.

At the screening visit, subjects in suspicion cohort are instructed and equipped to record physical activity (PA) using a triaxial UKK RM42 accelerometer (UKK Terveyspalvelut Oy, Tampere, Finland) over a seven-day period (Supplementary appendix 2.5). Subjects maintain a sleep diary for time they wore the accelerometer (Supplementary appendix 2.2.3)

Stool samples are self-collected by subjects at home between screening and biopsy visits, following detailed instructions (Supplementary appendix 2.3)

At the laboratory visit blood and baseline urine samples are collected at the laboratory of Turku University hospital (Supplementary appendix 2.3). In suspicion cohort, urine samples are collected at baseline and 1-3 days after prostate biopsies for bacterial culture and antimicrobial susceptibility testing.

Subjects in suspicion cohort undergo both biparametric prostate and body MRI examinations in the same scanning session (Supplementary appendix 2.4).

At the biopsy visit, subjects underwent standard clinical PCa diagnostics, including DRE, PSA and transrectal ultrasound (TRUS). After shared decision-making, subjects who proceeded with prostate biopsies comprised the final suspicion cohort. Biopsies consisted of systematic TRUS-guided 12-core biopsies with additional cognitive targeted biopsies of suspicious MRI-lesions (PI-RADS v2.1 score 3-5). Obtained samples are evaluated by an experienced uro-pathologist using the 2014 International Society of Urological Pathology Modified Gleason Grading System.

Treatment decisions are made according to local and international guidelines, in agreement with the patient. If the biopsy results are benign and the subject has BPH, if TURP is planned, he will be given opportunity to participate in benign cohort. If the subject is diagnosed with PCa and RALP is planned, he will be given opportunity to participate in cancer cohort.

2.1.2.2 Other cohorts

Subjects with 1) localized PCa planned for radical prostatectomy, 2) BPH planned for TURP or 3) bladder cancer planned for radical cystoprostatectomy, were screened for eligibility. At the screening visit, participants who provide written informed consent undergo study procedures, beginning with detailed questionnaires (Supplementary appendix 2.2). Anthropometric measurements (height, weight, BMI, and waist circumference) are recorded by the investigator.

Stool samples are self-collected by subjects at home between screening visit and planned surgical procedure following detailed instructions (Supplementary appendix 2.3).

At the laboratory visit blood and baseline urine samples are collected at the laboratory of Turku University hospital (Supplementary appendix 2.3).

Planned surgery is performed according to routine protocol. Routine diagnostic histopathological procedures will be followed, and study tissue samples are obtained in the department of pathology (Supplementary appendix 2.3.3).

Further treatment is planned according to local and international guidelines, in agreement with the patient.

2.2 Surveys

2.2.1 General data

A general study specific questionnaire covering medical history, medications, family cancer history, socioeconomic status, and lifestyle factors—including diet, physical activity (PA), sleep, bowel function, smoking, alcohol use, and endocrinological markers (e.g., baldness, ring-to-middle finger length ratio, and acne history) is filled in all cohorts. Anthropometric measurements (height, weight, BMI, and waist circumference) are recorded by the investigator.

2.2.2 Diet

Questionnaire determining the general eating/diet habits (e.g. use of spread, cereals, fruit) is collected at baseline in all cohorts. A validated 18-question Index of Diet Quality (IDQ) questionnaire is used to describe a health-promoting diet (22). Each question is scored, and the dietary quality is defined to be good if the scores were ≥10/15 (health-promoting diet) and poor if the scores were <10/15 (non-health-promoting diet). IDQ score reflects dietary intake of key foods and nutrients associated with health and depicts adherence to dietary recommendations, with significant correlations of higher IDQ and higher dietary intake of

protein, fiber, calcium, iron, vitamins and a higher ratio of unsaturated to saturated fatty acids and a lower intake of saturated fatty acids and saccharose. In addition to the IDQ, we also included questions related to foods and nutrients reported to be associated with either PCa or GM. These include xylitol, products containing lactic acid bacteria, soft drinks without sugar, red meat, sausage/cold cuts, poultry, beans, soy and tofu.

2.2.3 Sleep

Subjects in suspicion cohort keep a sleep diary during the seven nights they used the accelerometer. The following data is gathered after waking up in the morning: 1) time gone to bed; 2) number of times getting out of bed during sleeping; 3) wake up time as well as 4) perceived alertness (1=very alert….9=very sleepy). Subjects also grade the subjective quality of sleep with scale from 1-10.

2.2.4 Physical activity

Questionnaire determining PA is collected at baseline in all cohorts. The 6-question questionnaire assessed subjects` average regular weekly PA in terms of frequency, duration and type of activity as described below. The questionnaire was developed by the UKK Institute aiming to measure whether people fulfil the current recommendations for health-enhancing PA. It was used in population-based Health 2011 Study conducted by the National Institute of Health and Welfare in Finland.

1 = hardly any regular weekly PA

2 = Easy and peaceful aerobic type exercise (no perspiration or shortness of breath, for example light walking)

3 = Brisk and lively aerobic type exercise (some perspiration and shortness of breath, for example brisk walking)

4 = Vigorous and strenuous aerobic type exercise (profound perspiration and shortness of breath, for example jogging or running).

5 = Neuromuscular training (for example keep-fit circuit training or muscular strength training in a gym)

6 = Exercise that requires or improves balance (e.g. dancing, sports games, balancing exercises)

2.3 Samples

The study sample analysis plan is presented in Supplementary Figure 2.

2.3.1 Blood

Blood samples are collected in all cohorts. Routine clinical blood analyses conducted in the clinical hospital laboratory include PSA, serum testosterone, serum creatinine, estimated glomerular filtration rate and complete blood count. Serum samples for research purposes are used to measure steroid hormone concentrations and for global analysis of molecular lipids and polar metabolites by gas chromatography and plasma samples for steroid analysis by liquid chromatography. Whole blood is used for germline DNA sequence analysis by exome sequencing.

2.3.2 Stool

Stool samples with sample preserving medium are self-collected by subjects at home to three tubes in all cohorts, following detailed instructions. The tubes contain media that stabilizes

the microbiome at room temperature conditions for seven days. Samples are delivered or mailed to the microbiology laboratory at University of Turku, divided into aliquots and stored frozen in – 80 °C until prepared for further analyses. OMNIgeneGUT (DNA Genotek, Ottawa, Canada) tubes are used for microbiological shotgun metagenomic sequencing, OMNImetGUT (DNA Genotek, Ottawa, Canada) for gut metabolomics analyses with gas chromatography coupled to mass spectrometry or liquid chromatography mass spectrometry and eSwab (Copan Diagnostics, California, USA) for microbiological cultivation and antimicrobial susceptibility testing. eSwab contains liquid Amies transporting medium which elutes and preserves the sample for cultivation during transportation.

2.3.3. Tissue

Prostate biopsies obtained in the suspicion cohort are evaluated by an experienced uro-pathologist using the 2014 International Society of Urological Pathology Modified Gleason Grading System.

Fresh frozen tissue samples are collected from subjects going to surgery (TURP, RALP, cystoprostatectomy). Fresh tissue is collected in the the department of pathology and processed. Preoperative MRI images are utilized to retrieve core sampling from cancerous and benign areas from RALP specimen. Tissue samples are used for microbiota analysis with shotgun metagenomic sequencing, transcriptomics, and mass spectrometry analysis for metabolomics and steroids. For the global profiling of polar metabolites, gas chromatography coupled to time-of-flight mass spectrometry (GCTOFMS) is used. In addition to the traditional tissue metabolomics with gas chromatography-tandem mass spectrometry (GC-MS/MS), we utilize also spatial metabolomics.

2.3.4 Urine

Urine samples are collected at the laboratory of Turku University hospital at baseline in all cohorts for steroid and biomarker analysis. Urine sample is collected in suspicion cohort also 1-3 days after prostate biopsies for bacterial culture and antimicrobial susceptibility testing. Antimicrobial susceptibility is determined with phenotypic and genotypic methods from urine samples.

2.4 MRI

2.4.1 Imaging

Subjects in suspicion cohort undergo both prostate and body MRI examinations in the same scanning session. Subjects receive natriumpicosulphate drops (Laxoberon, Boehringer Ingelheim GmbH) and a Bisacodyl enema (Toilax, Orion Pharma Ltd) for bowel preparation. MRI examinations are performed using a 3T MR scanner (MAGNETOM Skyra Fit, Siemens Healthineers AG, Forchheim, Germany). Body array coils are used for image data acquisition. No endorectal coils are used. Prostate imaging included T2-weighted anatomical sequences in axial and sagittal planes, and diffusion-weighted imaging (DWI) using single-shot spin-echo echo-planar imaging. For body composition analysis, 2-point Dixon VIBE sequences were acquired, enabling quantification of fat and water images according to the AMRA® Body Composition Protocol. Prostate MR images are evaluated using a both Likert scoring system as previously reported (26): 1, significant cancer is highly unlikely to be present; 2, significant cancer is unlikely to be present; 3, significant cancer is equivocal; 4, significant cancer is likely to be present; 5, significant cancer is highly likely to be present, and PI-RADS 2.1 system (27).

2.4.2 Body composition analysis

Body composition analysis is performed using ABCDix: Analysis of Body Composition using Dixon-MRI by nine (IX) algorithms, a fully automated processing pipeline written using Python 3.12.0 that is developed in-house. The phases of the process are illustrated in Supplementary Figure 2. The two inputs are 1) Dixon fat image (Supplementary figure 3, Image 1) and 2) Dixon water image (Supplementary figure 3, Image 2); whereas the four outputs are normalized volumes of abdominal 1) SAT, 2) VAT, 3) MUS and 4) LFC. The runtime is approximately five minutes per subject. Algorithm 1 concerns segmentation of the trunk to exclude the arm. Algorithm 2 concerns localization of the abdominal cavity which is defined from cranial dome of liver to cranial end of caput femoris. Algorithm 3 concerns intensity inhomogeneity correction, followed by identification of the adipose tissue which is recognized by binarizing the fat image (Supplementary figure 3, Image 3) using Otsu's method. Algorithm 4 concerns the segmentation of SAT, which is defined as the largest singular adipose tissue surrounding the trunk (Supplementary figure 3, Image 4). Algorithm 5 concerns the segmentation of VAT, which is defined as any adipose tissue that is not SAT (Supplementary figure 3, Image 5). Algorithm 6 concerns binarization of the water image (Supplementary figure 3, Image 6) using Otsu's followed by generation of a mask Ω (Supplementary figure 3, Image 7) that encompasses VAT. The first part of MUS is then segmented by ignoring the skin and whatever that is inside Ω (Supplementary figure 3, Image 8). Algorithm 7 concerns segmentation of the second part of MUS i.e. the psoas muscle, by first locating the backbone and then identifying the pair in Ω which are attached to the left and right of the backbone (Supplementary figure 3, Image 9). Algorithm 8 concerns segmentation of liver, which is identified by the largest singular 3D structure (excluding MUS) on the right side of the binarized water image. Algorithm 9 concerns minor corrections and the final result generation. The segmentations of SAT, VAT, and MUS are first refined by filtering,

morphological operations, and majority voting to get rid of spurious i.e. small and isolated segmentations. Next, the volumes (in cm^3) of the SAT, VAT, and MUS are computed by multiplying the total number of voxels in each segmentation map (Supplementary figure 3, Image 10) by the voxel size (8.8 cm^3). They are then normalized by the length (cm) of the abdominal cavity and thus are expressed in cm^3/cm. LFC is defined by first calculating the fat signal fraction $\eta=F/(W+F)$ for each voxel in the segmented liver, where W and F are voxel intensities from the water and fat images respectively. Then, the median of η over the liver is taken as LFC. Dixon-measured LFC correlates strongly with MRI spectroscopy–derived LFC but consistently yields slightly higher values.[40]

2.5 Accelerometer

PA is recorded in suspicion cohort over one-week period with triaxial accelerometer (UKK RM42, UKK Terveyspalvelut Oy, Tampere, Finland). During waking hours, the accelerometer is attached to an elastic belt and worn on the right side of the hip for seven consecutive days, except during shower and other water activities. When going to bed, the accelerometer is moved from the belt to an adjustable wristband and attached to the non-dominant wrist on the knuckles' side. Participants receive both oral and written instructions about the accelerometer use and how to change the attachment point. The accelerometer collects and stores the raw tri-axial data in actual g-units in ±16G range at 100 Hz sampling rate.

During waking hours, the mean amplitude deviation (MAD) is calculated from the resultant acceleration signal in six-second epochs. The epoch-wise MAD values are converted to metabolic equivalents (3.5 mL/kg/min of oxygen consumption, MET). MVPA is defined as MET values higher than or equal to 3.0 (MAD over 91.5 mg). Light PA was defined as MET

values higher than or equal to 1.5 but less than 3.0 (MAD value between 22.5 and 91.5 mg) and moderate and vigorous physical activity (MVPA) as MET values higher than or equal to 3.0 (MAD over 91.5 mg).

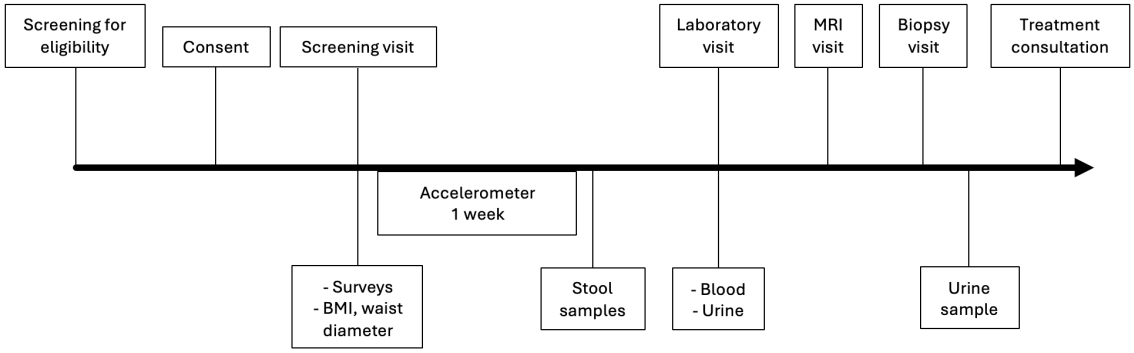
2.6 Supplementary tables and figures

Supplementary table 1. Univariate and multivariate analyses of the association between variables and prostate cancer. Data presented as median (interquartile range) or frequency (percentage).
PCa=Prostate cancer, BMI=Body mass index, PSA=Prostate-specific antigen, 5-ARI=5-alpha-reductase inhibitor, MVPA=Moderate and vigorous physical activity, SAT=Subcutaneous adipose tissue volume of the abdominal region, VAT=Visceral adipose tissue volume of the abdominal region

		Univariate		**Multivariate**		
Characteristic	**Benign (N=117)**	**PCa (N=181)**	**p-value[1]**	**OR[2]**	**95% CI[2]**	**p-value[2]**
Clinical variables						
Age (yr)	65 (59, 69)	69 (63, 75)	<0.001	1.06	1.02, 1.11	0.007
BMI (kg/m^2)	27.5 (24.9, 31.1)	27.1 (24.7, 30.6)	0.701	1.00	0.94, 1.06	0.986
PSA (ug/l)	7.9 (5.9, 10.0)	8.0 (5.8, 11.0)	0.415			
Free PSA (%)	16.4 (12.1, 20.1)	12.9 (9.0, 17.2)	<0.001			
Prostate volume (cm^3)	50 (38, 62)	35 (27, 48)	<0.001			
PSA density (ug/l/cm^3)*	0.12 (0.09, 0.16)	0.17 (0.13, 0.24)	<0.001	1.69	1.31,2.28	<0.001
Testosterone (ug/l)	14 (10.0, 17.0)	14.0 (10.0, 18.0)	0.964			
5-ARI	12 (10%)	29 (16%)	0.158	1.20	0.52,2.86	0.679
Physical activity and anthropometrics						
MVPA (h/week)	2.53 (0.69, 4.75)	1.29 (0.21, 3.59)	<0.001			
Steps (/day)**	5710 (3168, 7564)	4356 (2411, 6191)	0.004	0.92	0.83, 1.01	0.085
Sleep (hours/night)	6.20 (5.10, 7.10)	6.70 (5.50, 7.50)	0.027	1.18	1.01, 1.40	0.041
SAT (cm^3/cm)	1662 (1367, 2019)	1514 (1203, 2056)	0.193			
VAT (cm^3/cm)	1493 (1007, 1851)	1471 (857, 1959)	0.901			
Muscle volume (cm^3/cm)	1496 (1374, 1654)	1430 (1259, 1620)	0.009			
Liver fat content (%)	6.6 (4.8, 10.1)	6.4 (4.2, 9.6)	0.403			
Lifestyle factors						
Diet			0.055			
Health-promoting	52 (44%)	101 (56%)		ref	ref	-
Non-health-promoting	65 (56%)	80 (44%)		0.68	0.39, 1.17	0.159
Smoking			0.048			
Never	50 (42.7%)	80 (44%)		ref	ref	-
Former	43 (36.7%)	81 (45%)		1.14	0.64, 2.03	0.648
Active	24 (20.5%)	19 (11%)		0.57	0.23, 1.35	0.200
Family history of PCa	22 (19%)	38 (21%)	0.645	1.82	0.93, 3.70	0.088
Acne			0.054			
Never	69 (60%)	129 (72%)				
Teenage	42 (37%)	48 (27%)				
Adulthood	4 (3.5%)	2 (1.4%)				

[1]Wilcoxon rank sum test; Pearson's Chi-squared test; Fisher's exact test, [2]Binomial logistic regression model
*In multivariate analysis, PSA density is scaled per 0.1 units (tenths) to enhance interpretability of OR and 95%CI
**In multivariate analysis, steps are scaled per 1000 steps (steps/1000) to improve interpretability of OR and 95%CI

Supplementary figure 1. Study visits and procedures for suspicion cohort



Supplementary figure 2. Promic study sample analysis plan.

Blood Hospital laboratory	Plasma 9 x 1 ml	Metabolomics
	Serum 8 x 1 ml	Steroid analysis
	Whole blood 1 x 9 ml	Germline DNA sequencing

Stool Subject collects	OMNIgeneGUT	Microbiota shotgun sequencing
	OMNImetGUT	- Metabolomics - Steroid analysis
	Fecal swab	- Bacterial culture - Antimicrobial susceptibility

Tissue Surgeon/pathologist	Fresh frozen tissue	- Microbiota shotgun sequencing -Metabolomics

Urine Hospital laboratory	Frozen aliquots	- Steroids
	Fresh urine	- Bacterial culture - Antimicrobial susceptibility

Supplementary figure 3. Presentation of the MRI-based body composition analysis process.