Physiological and metabolic effects of healthy female aging on exhaled breath biomarkers

Highlights
Physio-metabolic effects of female aging are reflected in breath VOC markers
Overall VOC expressions were suppressed in adults under oral contraceptive pills
Young homosexual/lesbian adults were breathomic outliers
Clinical interpretations of breath VOCs as biomarker, must consider age effects

Healthy females (age: 07 – 80 years)
Physiological and metabolic effects of healthy female aging on exhaled breath biomarkers

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SUMMARY
Healthy aging driven physio-metabolic events in females hold the key to complex in vivo mechanistic links and systemic cross talks. Effects from basic changes at genome, proteome, metabolome, and lipidome levels are often reflected at the upstream phenome (e.g., breath volatome) cascades. Here, we have analyzed exhaled volatile metabolites (measured via real time mass spectrometry based breathomics) data from 204 healthy females, aged between 07 and 80 years. Age related substance-specific differences were observed in breath biomarkers. Exhalation of blood-borne endogenous organosulfur, short-chain fatty acids, alcohols, aldehydes, alkene, ketones and exogenous nitriles, terpenes, and aromatics have denominated interplay between endocrine differences, energy homeostasis, systemic microbial diversity, oxidative stress, and lifestyle. Overall marker expressions were suppressed under daily oral contraception. Young homosexual/lesbian adults turned out as breathomic outliers. Previously proposed disease-specific breath biomarkers should be reevaluated upon aging effects. Breathomics offers a noninvasive window toward system-wide understanding and personalized monitoring of aging i.e., translatable to gerontology.

INTRODUCTION
Biological aging is an everlasting, inevitable, and irreversible process that leads life toward death. Being the basic balancing act of our mother nature, aging eventually brings along certain life events, health conditions, and diseases, which we have been trying to understand since the dawn of biomedical science.

Among us, female aging consists of the most complex and intriguing physio-metabolic phenomena, which summons research that culminates in basic, clinical, and translational science beyond the boundaries of conventions. While considering the putative regulating factors, i.e., endocrine signaling and interplay with metabolic and hormone-receptor pathways (Burger et al., 2007; Russell and Kahn, 2007), energy homeostasis (Borie et al., 2014; Roberts and Rosenberg, 2006), oxidative stress (Romano et al., 2010), and physiological floral (e.g., gut microbiota) diversity/activity (DeJong et al., 2020; Wilmanski et al., 2021) are the prime determinants of both premenopausal and postmenopausal aging (Elliott et al., 2021). To understand the effects of aging driven endocrine–homeostatic–gut-microbial cross talks (Clarke et al., 2014; Marciano and Vajro, 2017; Vitale et al., 2013; Zhang et al., 2021) on the in vivo physiology, metabolism, and biochemistry, we seek unique, interdisciplinary, and intelligible approaches. Although key changes (Whittemore et al., 2019; Zhu et al., 2019) take place at the genome level (e.g., increased prooxidants/decreased antioxidant driven telomere degradation) (Reichert and Stier, 2017), their reflections are anticipated and often observed at the upstream cascade e.g., on proteome, metabolome, lipidome, and/or volatome.

High-resolution and real time mass spectrometry based noninvasive, rapid, and repeated evaluation of exhaled volatile metabolites offers immediate overview of various physio-metabolic (Spacek et al., 2018; Sukul et al., 2014, 2015, 2017a), biochemical (Refat et al., 1991; Sukul et al., 2021; Weber et al., n.d.) and pathological/therapeutic processes (Löser et al., 2020; Paardekooper et al., 2017; Trefz et al., 2019) via breathomics. Previously we have demonstrated the effects of natural menstrual cycle and oral contraception on the exhaled volatile organic compounds (VOCs) profile from healthy adults (Sukul et al., 2018). Effects of well-known endocrine and homeostatic changes on different metabolic pathways such as
glycolysis, lipolysis, protein catabolism, and cholesterol homeostasis etc. were attributed to the expression of putative volatile byproducts in exhaled breath.

Therefore, the comprehensive volatile profiling of female aging may address many long-standing questions and/or unknown aspects upon the basic biochemistry of aging at the cellular level and could help to translate those for clinical implications toward the wellbeing of our aging society. Such a volatome map of female aging should incorporate the most crucial life events such as childhood, puberty, adolescence, reproductive womanhood (+oral contraception), menopause, and postmenopausal life.

Within this study we have pioneered the apprehensive breath biomarkers print of prime aging period in women, ranging from the age of 07–80 + years. Our analysis incorporates both new and existing large datasets, which originated via the same methodology, analytical techniques, and under the same operational conditions. Readers will be able to relate the endogenous and blood-borne volatile marker expressions in exhaled breath during various spans of healthy womanhood. The expanding importance and interest of noninvasive and rapid phenotyping of female aging and aging related life events and health conditions are addressed.

RESULTS

Distinct differences are observed in exhaled breath biomarker profiles in different age groups (Demographic data are listed in Table 1). Blood borne VOCs with various endogenous/exogenous origin and/or physicochemical properties behaved differently. While looking at the expression of different substance classes, such as sulfides, acids, alcohols, aldehydes, alkene, ketones, terpenes, and aromatics, the substance specific effects of aging are perceivable upon factors such as endocrine differences, biochemical homeostasis, systemic microbial diversity, physio-metabolic states, and lifestyle/environment. Pronounced suppression of VOC exhalation is observed under the presence of oral contraceptive pills in adults. Normal physio-metabolic variations of substances are reflected by the coefficient of variations in different groups. Premenopausal adults with homosexual orientation turned out as complete breathomic outliers.

Relative differences in alveolar abundances and relative standard deviations of VOCs within different age groups

Figures 1A and 1B represent heat maps of relative differences in exhaled alveolar abundances and relative standard deviations (RSDs) of 23 selected VOCs from 204 female subjects. Mean (age group wise) of normalized (onto corresponding maximum) VOCs are expressed here. Selection criteria of VOCs are described in the methods section. Relative differences are observed within different age groups. Differences and overlaps of volatomic patterns among the study groups are presented in supplement (Figure S1).

Comparison of exhaled alveolar abundances of endogenous and exogenous VOCs within different age groups

Absolute alveolar abundances of exhaled VOCs in different age groups are presented as boxplots in Figures 2A–2C. Here, (A) represents the sulfides and smoking habit/environmental exposure related VOCs, (B) represents the oxygenated substances such as aliphatic alcohols, acids, and ketones, and (C) represents the aliphatic aldehydes, unsaturated hydrocarbons, and terpene. The mean (with corresponding standard deviation) and median (along with corresponding maximum and minimum) alveolar abundances of these substances are presented in supplement (Table S1).

From all pairwise comparisons, the differential expressions in exhaled VOCs between age groups are indicated with respect to the ‘Preteen (Y: 7–12)’ group and also with respect to the ‘Adults on oral contraceptive pill/OCP (Y: 20–49)’ group. Detailed data on mean expiratory abundances of VOCs and SE of the mean (SEM) in each age group along with all statistical comparisons (with corresponding p values) are presented in supplement (Table S2 and S3).

Correlations between selected VOCs of interest and age

The correlation coefficients and respective p values between VOCs of interest and age are presented in Table 2. Detailed inter-VOC correlations (with respect to aging as the independent denominator) along with corresponding p values are presented in supplement (Table S4).
Exhaled VOC abundances within three groups of adults

Figure 3 represents the expressions of exhaled VOC abundances within three groups of adults (Y: 20–49) viz. Naturally menstruating heterosexual adults (n = 15), heterosexual adults undertaking daily OCP (n = 15) and homosexual/lesbian adults (n = 3). Owing to low occurrence of homosexual females, statistical comparisons were not performed, herein.

DISCUSSION
Organosulfur

Understanding the varying expression of the natural flora throughout the human lifespan and aging Ragonaund and Biragyn, 2021; Wilmanski et al., 2021; Xu et al., 2019; Zhang et al., 2021) is of high scientific and clinical importance Bosco and Noti, 2021; Lee and Hase, 2014; Price and Nitabach, 2021; Romano et al., 2021). Most importantly, the gut microbiota (aka. The forgotten organ) plays a crucial role in endocrine homeostasis Rastelli et al., 2019) and thereby, intermediately may affect many metabolic pathways, which are closely related to aging and longevity (Wilmanski et al., 2021). Volatile organosulfur in exhaled breath are potentially sourced by in vivo bacterial emissions i.e., mainly from the gut microbiome (Tangerman, 2009). While dimethyl sulfide (C2H6S) and allyl-methyl sulfide (C4H8S) are constantly originated via the anaerobic bacterial methylation in the gut (Tangerman and Winkel, 2007), hydrogen sulfide (H2S) and butanethiol/methyl-propyl sulfide (C4H10S) are predominantly produced by the oral and nasal cavity bacteria, respectively (Sukul et al., 2017a). A recent metagenomic analysis has demonstrated that greater microbial diversity and abundances of several species are observed in premenopausal women that positively affect host metabolism (Zhang et al., 2021). Here, significantly increased H2S exhalation during preteen and teenage and elevated C4H10S in premenopausal adults (including those undertaking contraceptive pill) indicates overexpression of sulphur-reducing bacteria (or activity). This agrees with the proposition that free/active thiol (-SH) group in these organosulfur (aka. gasotransmitter) (Linden, 2014) facilitates endocrine regulation (e.g., sex hormone interplay) and maturation during preteen, teenage, and adulthood via the hypothalamic-pituitary-adrenal-gonadal axis (Mancuso et al., 2010; Zhu et al., 2011). We witnessed that generalized origins/sources of exhaled organosulfur greatly differ with age and their expression patterns can indicate mechanistic links between microbial-endocrine axis and host’s metabolic output during different spans of aging.

Short-chain fatty acids (SCFAs)
Similar to organosulfur, SCFAs e.g., acetic acid (C2H4O2), butyric acid (C4H8O2), and propionic acid (C3H6O2) are produced via breakdown of dietary fibers and starch by gut bacteria (Silva et al., 2020; Wang et al., 2020). Differences in absolute abundances of these volatile SCFAs in breath reflect previous observations in various tissues (Wang et al., 2020). The pronounced increase in acetic acid during teenage indicates effects from changes in dietary habits (De Angelis et al., 2020; Winpenny et al., 2018) and under increased production of vaginal lubrication and acidic protection against bacterial vaginitis (Boskey et al., 1999; Chaudry et al., 2004).

Table 1. Anthropometric information of subjects

| Participant groups | Gender | Numbers (n) | Age range (Years) | BMI range (Kg/sq.m) | Smoker | Alcoholic | Special Diet | Acute, Chronic Disease/Medication | OCP | Pregnancy/Expecting |
|--------------------|--------|-------------|-------------------|--------------------|--------|-----------|-------------|-------------------------------|-----|--------------------|
| Preteen            | F      | 19          | 07 to 12 (07–18)  | N/A                | No     | No        | No          | Yes (n = 09)                  | N/A | N/A                |
| Teenage            | F      | 35          | 13 to 19 (15–29)  | No                 | No     | No        | No          | No                            | Yes (n = 11) | No |
| Adults             | F      | 33          | 20 to 49 (17–30)  | Yes (n = 09)       | No     | No        | No          | No                            | Yes (n = 15) | Yes (n = 15) |
| Postmenopausal     | F      | 37          | 50 to 59 (21–34)  | Yes (n = 11)       | No     | No        | No          | No                            | No | N/A                |
| Postmenopausal     | F      | 47          | 60 to 69 (20–36)  | Yes (n = 02)       | No     | No        | No          | No                            | No | N/A                |
| Postmenopausal     | F      | 36          | 70 to 80 (18–35)  | Yes (n = 02)       | No     | No        | No          | No                            | No | N/A                |

Number of female subjects in each group, age range and body mass index (BMI) range are listed along with life style attributes e.g., cigarette smoking or alcohol drinking or any special dietary habits and clinically important parameters e.g., use of birth control pills, pregnancy, any health condition, or medication etc.
Aldehydes: Age-related shortening of chromosomal telomeres and associated DNA damage downregulates body’s antioxidant defense (Flor and Kron, 2016). Aliphatic aldehydes (\(a, b\)-unsaturated) are well-known to be produced...

Figure 1. Relative differences in normalized mean alveolar abundances (A) and in mean relative standard deviations (B) of selected VOCs from all subjects within different age groups

(A) Y axis represents the protonated VOCs of interest and X axis represents the phases of female aging. VOCs were tentatively identified based on their mass/charge ratio. VOC data from each individual were normalized onto corresponding maximum (of all subjects) values of substances. The mean (/age group) of those normalized values are presented within each age group. OCP refers to ‘oral contraceptive pills’. Red and blue colors symbolize relatively higher and lower abundances, respectively.

(B) Y axis represents the protonated VOCs and X axis represents the age groups. RSD values were normalized onto the corresponding maximum values from each substance. Light and dark colors symbolize relatively higher and lower coefficients of variation, respectively.

Aldehydes

Age-related shortening of chromosomal telomeres and associated DNA damage downregulates body’s antioxidant defense (Flor and Kron, 2016). Aliphatic aldehydes (\(\alpha, \beta\)-unsaturated) are well-known to be produced...
under such conditions of oxidative stress e.g., via lipid peroxidation (Grimsrud et al., 2008). Here, pentanaldehyde (C5H10O), butyraldehyde (C4H8O) and crotonaldehyde (C4H6O) exhalations are elevated in postmenopausal group, which indicates the increased oxidative stress/reduced antioxidative defense in elderly women. Based on these findings, results from previous studies describing even less pronounced differences in aldehyde abundances as biomarkers for detection of respiratory diseases (Bartoli et al., 2011; Corradi et al., 2003) and cancers (Fuchs et al., 2010; Poli et al., 2010) should be reevaluated with respect to potential age effects.

**Diol**

Propanediol (C3H8O2) closely mirrored the profiles of abovementioned aldehydes and with good correlations (Table S4). Studies have indicated that certain glycolipids undergo α-oxidation or ω-oxidation (Rizzo, 2014), which gives rise to such glycol molecules (Glycolipids - an overview | ScienceDirect Topics, n.d.). Therefore, we propose propanediol to be a tentative volatile marker for postmenopausal aging related oxidative stress, which need to be validated in a large independent cohort before being termed as biomarker.

**Alcohol**

In contrast to that, endogenous ethanol (C2H6O) behaved similar (with good correlation) to organosulfur exhalations, which indicates its anticipated origin from the carbohydrate metabolizing bacteria in the intestine (Elshaghabee et al., 2016; Logan and Jones, 2000). Being the primary source of the body’s energy expenditure, carbohydrate metabolism increases during the developmental (physiological maturation) stages and reaches its maximum at the reproductive adulthood. Carbohydrates are broken down to glucose in the small intestine and ethanol is known to increase the intestinal epithelial and colon permeability to facilitate glucose transport for hepatic and cellular glycolysis (Glycolysis - an overview | ScienceDirect Topics, n.d.). Presence of high estrogen reduces intestinal ethanol metabolism (Kwa et al., 2016; Zeiner and Kegg, 1981). Therefore, the increased alveolar elimination of endogenous ethanol in premenopausal adults (without contraception) can be attributed to both its increased production and decreased metabolic breakdown. Owing to profound drop in sex hormones after menopause along with decreased intestinal microbial activity (Elahi and Muller, 2000; Roberts and Rosenberg, 2006), ethanol exhalation also declines with postmenopausal aging. There is evidence to support the claim that contraception causes dysbiosis of oral and GI microbiome (Sukul et al., 2018), which explains the downregulation of endogenous ethanol production as well as its increased metabolism (via synthetic estrogen) in adults undertaking contraceptive pills.

**Ketone**

Owing to elevated endogenous ethanol (facilitating intestinal glucose transport), one may assume an increased glycolysis in adults (without pill) and thereby higher breath acetone (C3H6O) as a putative byproduct (Kalapos, 2003). Unexpectedly, we observed significant decrease in alveolar acetone abundances with high physiological variation in adults (without pill). Given the fact that we have considered data from the ovulation phase (in all adults), corresponding lowering of circulating natural steroid hormones lowered glucose metabolism (Sukul et al., 2018). Estrogen (+receptors) mediates cytoplasmic kinase (Chen et al., 2009) to modulate glycolysis and progesterone mediates lipolysis in fat compartments by suppressing insulin activity (Kalhoff, 1982). Thus, daily intake of supplementary sex hormones for contraception resulted in relatively higher acetone exhalation with low physiological variation (i.e., endocrine steady state). Therefore, the endogenous ethanol seems to act as a negative feedback (gut permeability versus intestinal transport to blood) signal for cellular glycolysis.

**Hemiterpene**

Previous studies have indicated an inverse relationship between endogenous breath isoprene (C5H8) abundances and aging (Kushch et al., 2008; Lechner et al., 2006) in males. Here, we observed effects of female...
aging and endocrine steady state (under contraception) on exhaled isoprene. Although C5H8 was believed to be a product of the mevalonate pathway (cholesterol biosynthesis) (Stone et al., 1993), our latest breathomic findings on >1000 subjects in association with lipid profiling and genetic analysis has disregarded the putative in vivo source (Sukul et al., 2021). Here, the overall C5H8 expressions in premenopausal (i.e., gradual increase from preteen to adulthood) and postmenopausal (i.e., maximum increase just after the menopause and then gradual decrease with further aging) women cohorts indicate its relation to sex hormone regulation i.e., estrogen-progesterone interplay. In line with the 'biogenetic isoprene rule' (Hillier and Lathe, 2019), we believe that the C5 isoprene units are utilized as basic building blocks for higher terpenoids, steroids, and sex hormones (Tong, 2013). Thus, increase in endogenous C5H8 from preteen until adulthood depicts an obvious increase in sex hormone production to apprehend the reproductive development and natural menstrual cycle demand. Consequently, such increase was not observed in adults undertaking contraceptive pills as the biological need is supplemented in them via daily synthetic sex hormones intake. Menopause and estrogen deficiency come along with an increased risk for endothelial dysfunction and a decline in vasodilatory function between 49 and 60 years (Taddei et al., 1996) under reduced bioavailability of nitric oxide (NO) (Yu et al., 2010) in vascular smooth muscle compartments. This leads to vasoconstriction and causes smooth muscle contraction (Somani et al., 2019). As minute muscle contractions (even during sleep) immediately produce isoprene (King et al., 2012), the maximum increase in C5H8 exhalation within the immediate postmenopausal cohort indicates the onset and progression of vascular aging. Despite one may think of oxidative stress as a parallel denominator, we could not find any reasonable correlations with oxidative stress markers (viz. aldehydes and propanediol).

Aliphatic nitrile, aromatics, monoterpen, cyclic ketone, and carboxylic acids

Exogenous substances such as acetonitrile (C2H3N), benzene (C6H6), toluene (C7H8), acrolein (C3H4O), and furan (C3H4O) are mainly associated with smoking and/or previous exposure. Limonene (C10H16), cyclopentanone (C5H8O), crotonic acid (C4H6O2), and formic acid (CH2O2) are most probably sourced from oral/dermal intake, for example, from beverages (e.g., orange juice), mouthwash/mint, food flavors/preservatives, and cosmetics/disinfectant/cleaning agents (Sukul et al., 2017a). Some of these substances are, therefore, only present in subjects with smoking and/or specific lifestyle habits and remain independent of aging related effects. As furan may be stored in and released from fatty compartments (depending on respiratory and haemodynamic factors), the exhaled profiles differed from other smoking related substances.

Significant downregulation of almost all VOCs under the intake of daily supplementary sex hormones indicates the prominent role of endocrine interplay on in vivo metabolic processes. Juster et al. reported the elevated testosterone and progesterone levels in lesbian adults than that in age matched heterosexual female cohort (Juster et al., 2016). Further to that, a positive association of oestradiol levels is observed between heterosexual men and lesbian/bisexual women. In our results, the overall heterogeneous (to other

| VOCs            | Correlation coefficient (R) | p Value | Aging p Value | Correlation coefficient (R) | VOCs            |
|-----------------|-----------------------------|---------|---------------|-----------------------------|-----------------|
| Isoprene        | 0.328                       | 0.000   | 0.077         | -0.101                     | Crotonic Acid   |
| Cyclopentanone  | -0.006                      | 0.466   | 0.000         | 0.282                      | Butyric Acid    |
| Pentanal        | 0.599                       | 0.000   | 0.038         | -0.125                     | Propionic Acid  |
| Butanal         | 0.167                       | 0.009   | 0.000         | -0.296                     | Acetic Acid     |
| Crotonaldehyde  | 0.219                       | 0.001   | 0.004         | -0.185                     | Formic Acid     |
| Acrolein        | -0.047                      | 0.252   | 0.001         | -0.229                     | Acetone         |
| Ethanol         | -0.407                      | 0.000   | 0.299         | -0.037                     | Limonene        |
| Propanediol     | 0.443                       | 0.000   | 0.001         | 0.209                      | Furan           |
| Hydrogen Sulfide| -0.643                      | 0.000   | 0.238         | 0.05                       | Acetonitrile    |
| DMS             | 0.124                       | 0.039   | 0.290         | 0.039                      | Benzene         |
| AMS             | 0.246                       | 0.000   | 0.131         | 0.079                      | Toluene         |
| Butanethiol/MPS | -0.015                      | 0.418   | Age           |                            |                 |

Correlation coefficients (R value) along with corresponding p values are presented. Statistically significant (p value ≤ 0.005) correlations are assigned in bold.
adult) VOC exhalations in three lesbians (clustered together) indicate a different endocrine milieu under homosexual orientation. Observed similarities and dissimilarities between homosexual and heterosexual adults (with and without contraceptive pill) upon certain endogenous VOC expressions indicate unattended chapters of endocrine–metabolic–systemic microbial–biochemical homeostatic cross talks and thereby, shouts for further large-scale investigations. The expression patterns of endogenous organosulfur(s), SCFAs, alcohols, aldehydes, and alkene etc. Within/between premenopausal heterosexual (with and without contraception) and premenopausal homosexual adults with respect to postmenopausal adults indicates many interdependent mechanisms, which would enhance our understanding of menopause driven physio-metabolic complications e.g., endocrine disbalance, compromised bone metabolism, osteopenia, osteoporosis, and increased stress etc.

Real time VOC profiling of healthy female aging enables us to explore physio-metabolic attributes of this complex biological process. Aging driven expressions of endogenous VOCs have depicted a number of intermediate effects/influences from intrinsic and extrinsic factors. Interdependencies of intrinsic denominators e.g., natural endocrine regulation (+contraceptives), oxidative stress, energy homeostasis, and in vivo bacterial metabolism along with corresponding variations and cross talks are reflected in breath profiles. Understanding of mechanistic links between those biological cascades will offer new windows toward probiotic/therapeutic targets for aging related health conditions. In this observational study, we have analyzed mass spectrometry based breathomic data from >200 healthy female subjects to observe differences in VOC profiles with respect to aging and related normal/healthy life-events (e.g., puberty, teenage, adulthood, menopause, etc.). Given the facts that breath is a highly-dynamic matrix and the even putative/potential endogenous origins of even most abundant VOC biomarkers are not yet certain and largely debatable (Sukul et al., 2021) –the detection of changes in exhaled concentrations/profiles of the identifiable/quantifiable markers related to aging and potentially linked to physio-metabolic effects of aging, pathophysiology of aging, life-events, and/or drug effects was the major focus. Our results are indicating that there are major changes in the VOC profiles during aging. The observations offer fundamental prerequisites for breathomics based monitoring of female aging and aging related disorders. Therefore, any clinical study that is applying breathomics to investigate differences between patient cohorts should consider physio-metabolic effects from the subject’s age. At this early stage of research, however, it is not possible to derive “conclusive patterns of breath volatile metabolites, which are associated with the healthy aging in females” or to define (clinically) applicable “biomarkers of healthy aging”. Defining applicable biomarkers for aging and assessing sensitivities/specificity of such marker(s) require validation of potential marker(set) in independent patient cohorts. Our findings are translatable to geriatrics and gerontology to noninvasively monitor the course of healthy aging and/or related health conditions.

Limitations of the study
We have included breathomics data from female subjects only/mainly from German/European ethnicity and this may reflect differently in other ethnicities (because of obvious difference in downstream biological cascades). Comparison with age and gender matched male population is missing in our present approach. Although we have included a large number of subjects (considering our academic research perspective) and conducted repeated measurements, the overall expression of our observation may vary upon the entire population. As we came across only three homosexual individuals, observed physio-metabolic differences because of sexual orientation cannot be generalized. Our field of Breathomics is still at its infancy and standard method generated population data/resource on breath VOCs is missing and therefore, we could not conduct a population-based comparison of our findings.

STAR+METHODS
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Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.103739.

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AUTHOR CONTRIBUTIONS

P.S., J.K.S. and W.M. conceived the idea and planned the study. S.G., C.B., P.S. and P.T. recruited volunteers and performed experiments. P.S. and P.T. analyzed data. P.S. prepared the results and performed statistical analysis. P.S., J.K.S., D.C.F. and T.M. contributed to clinical interpretation and discussion. W.M. and P.T. contributed to analytical interpretations. PS wrote the manuscript, which was reviewed and edited by all authors. Correspondence and requests for materials should be addressed to P.S. (email: pritam.sukul@uni-rostock.de).

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR METHODS

KEY RESOURCES TABLE

| REAGENT or RESOURCE                      | SOURCE                     | IDENTIFIER                                      |
|------------------------------------------|----------------------------|-------------------------------------------------|
| Deposited Data                           |                            |                                                 |
| Exhaled VOC data from pre-teens and teens| Trefz et al. (2019)        | https://doi.org/10.3390/jcm8111797               |
| Exhaled VOC data from premenopausal adults| Sukul et al. (2018)        | https://doi.org/10.1038/s41598-018-29221-z       |
| Software and Algorithms                  |                            |                                                 |
| ToFDaq Viewer                            | TOFWERK                    | https://www.tofwerk.com/software/tofdaq/         |
| Breath Tracker, MATLAB v7.12.0.635, R2011a| MathWorks                 | https://www.mathworks.com/products/matlab.html   |
| SPSS Software v27                        | IBM                        | https://www.ibm.com/analytics/spss-statistics-software |
| SigmaPlot v14                             | SYSTAT                      | http://www.systat.de/SigmaPlot_Produkteite.html  |
| Unscrambler X software v10.3             | CAMO Analytics             | https://www.aspentech.com/en/acquisition/camo-analytics |
| Other                                    |                            |                                                 |
| PTR-ToF-MS 8000 real-time Mass-Spectrometer| IONICON Analytik       | https://www.ionicon.com/                        |

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources should be directed to and will be fulfilled by the Lead Contact, Pritam Sukul (pritam.sukul@uni-rostock.de).

Materials availability
This study did not generate new unique reagents.

Data and code availability
- The data are available upon reasonable request by contacting the lead contact.
- No new code was generated during the course of this study.
- Any additional information required to reanalyse the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Healthy female subjects
As we wanted to observe the physio-metabolic effects of pre- and post-menopausal female aging on breath biomarker profiles, exhaled VOC data from 204 healthy female subjects (aged between 5 and 85 years) were included for analysis from three subsequent clinical studies (viz. performed under same methodology and analytical parameters/conditions), conducted by us at the university hospital between 2013 and 2020. All clinical experiments were conducted according to the amended Declaration of Helsinki guidelines and signed informed consent from every healthy female subject were obtained in these studies (Approval number: A2012–0103, A2015–0008 and A2019-0040 – issued by the Institutional Ethics Committee of University Medicine Rostock, Germany). For children (below the age of 18), written consent from both parents were obtained. Thus, our analysis incorporates both new and existing data. Here the new data from healthy post-menopausal cohort is incorporated from recently accomplished study (A2019-0040) on bone aging and the existing data from premenopausal cohort is incorporated form our previously investigated volunteers (i.e. only healthy controls) during continuous glucose monitoring (A2012-0103) in children (Trefz et al., 2019) and breathomics of adults (A2015-0008) under menstrual cycle and contraception (Sukul et al., 2018). Subjects were not suffering from any acute or chronic diseases or were not undertaking any special diet or therapy.
**Determination of sample size for analysis**

We applied the analysis of variance (ANOVA) test for calculation of sample size. For a minimum detectable difference in mean substance intensities of 500 cps, an estimated standard deviation of 300 and 7 experimental groups to attain an alpha value of 0.005 and a test power of 0.99 while considering a population of 100,000, the sample size resulted at 200 (with minimal group size of at least 15 in each). Here, we have included 215 subjects for analysis in order to detect even less than 5% differences in exhaled VOCs up to low parts per trillion by volume (pptV) levels.

Eight subjects (three below the age of 7 and five above the age of 80 years) were excluded from data analysis as they had significantly hyperventilated (via respiratory rate >20/min). Rest of the subject’s anthropometric data are presented in Table 1.

Out of the above cohort, 204 subjects were included in final statistical analysis as three adults with homosexual orientation (without contraception) were excluded from the statistical analysis as they clustered as complete outlier from their age-matched cohort. Nevertheless, the differential expression of VOCs from these three adults with respect to the heterosexual adults (with and without contraception) are presented separately in Figure 3.

**Breath sampling protocol**

Each participant maintained a normal sitting position (Sukul et al., 2015) and performed oral breathing via custom made Teflon-mouthpiece of 2.5 cm diameter (Sukul et al., 2017b). Volunteers rested by sitting for 10–15 min before actual sampling. The transfer-line of PTR-ToF-MS was connected to the mouthpiece in order to continuously record VOC abundances in real-time. Mouthpieces were sterilized for reuse. Subjects inhaled and exhaled only via mouth (Sukul et al., 2017a) and followed our standard state-of-the-art breathing protocol i.e. established to significantly reduce the immediate intra- and inter-individual physiological/ventilatory variations (Sukul et al., 2020) and also to minimize the influences of the oral cavity via precedent wash-out. Measurements were repeated in subjects on different days. In each post-menopausal adult, measurement means derived from two simultaneous experiments that were repeated with an interval of a month. Similarly, in each pre-menopausal adult (with and without contraception), measurement means are calculated over two consecutive ovulation phases as the obvious endocrine and metabolic differences under the administration of daily supplementary sex-hormones (via birth-control pills) with that of natural menstrual cycle (Sukul et al., 2018), are to be considered.

**METHOD DETAILS**

**PTR-ToF-MS measurements of breath VOCs**

Breath VOCs were measured continuously via a PTR-ToF-MS 8000 (Ionicon Analytik GmbH, Innsbruck, Austria) and with pre-optimized experimental conditions (Herbig et al., 2009; Sukul et al., 2014), i.e. continuous side-stream mode of sampling via a 6 m long heated (at 75 °C) silco-steel transfer-line connected to a sterile mouthpiece. We used 20 mL/min of continuous sampling flow and the time resolution of the PTR-ToF-MS measurements was 200 ms. Thus, data points were generated after every 200 ms and on each data point hundreds of compounds were measured at their trace abundances (in both expiratory- and room air). The ion source current was set to 4 mA and the H2O flow was set to 6 ml/min. Drift tube temperature were set at 75 °C, voltage was 610 V and the pressure was 2.3 mbar. The resulting E/N ratio was 139 Td. After every minute a new data file was recorded automatically and the mass scale was recalibrated after each run (60s). We used the following masses for mass calibration: 21.0226 (H3O+-Isotope), 29.9980 (NO+) and 59.049 (C3H6O).

**VOC data processing**

VOCs were measured in counts per seconds (cps) and corresponding intensities were normalised onto primary ion (H3O+) counts. As PTR-MS continuously records both exhaled breath and inhaled room-air, we applied the ‘breath tracker’ algorithm (based on Matlab version 7.12.0.635, R2011a) to identify expiratory (i.e. alveolar/end-tidal phase) and inspiratory phases (Sukul et al., 2014). Here, we used acetone as the tracker mass as it is an endogenous substance, which has significantly higher signal intensity in expiration than in inhalation. As the high mass resolution of PTR-ToF-MS (4000–5000 Da/m) can assign volatiles upon their measured mass and corresponding sum formula with high precision (Sukul et al., 2017a), compound names are used while discussing results. VOCs are quantified via multi-component mixture of standard
reference substances. Quantification process under adapted sample humidity (as in exhaled breath) using a liquid calibration unit (LCU, Ionicon Analytik GmbH, Innsbruck, Austria) is our pre-established state-of-the-art (Trefz et al., 2018).

**Selection of VOCs for analysis**
Among hundreds of measured VOCs, here we considered compounds with expiratory abundances significantly above the inspiratory/room-air abundance. Out of those markers we selected 23 substances that are important breath biomarkers in clinical breathomics and reflect different origins, physico-chemical characters and dependencies on physiology, metabolism, pathology, therapy and lifestyle/habits (Löser et al., 2020; Sukul et al., 2017a, 2018; Trefz et al., 2013, 2019).

**Assignment of age groups**
Considering the ‘wide range of normal’ in children, girls may enter early puberty (i.e. physical maturity) by the age of seven (Early or delayed puberty, 2018) and then to early adolescence and menstrual cycle at around 12 years (Starting your periods, 2018). Since the beginning of teen age until mid or even late 40s is regarded as reproductive womanhood, while they remain fertile and/or sexually active. Around the age of 50 (ranges between 45 and 55 years) (Menopause, 2017) menopause takes place and they enter the post-menopausal life. This incepts numerous health complications (aka. post-menopausal syndrome) (Davis et al., 2015) viz. vasomotor symptoms (hot flashes, restlesslessness and anxiety), depression, insomnia, urogenital atrophy, gradual loss of bone mineral density (osteopenia and osteoporosis) as well as cardiac and cognitive dysfunctions etc. Therefore, to apprehend the above aspects, we have divided the study population in seven groups; namely: children at early/normal pre-teen (7–12 years), teenage (13–19 years), two premenopausal adults (20–49 years) i.e. with and without oral contraceptive pills (OCP) and three post-menopausal groups (50–59, 60–69 and 70–80 years). Above indicated life events were confirmed by participants during inclusion.

**QUANTIFICATION AND STATISTICAL ANALYSIS**
Analytical mean values (from all participants) of VOC abundances of each participant were calculated over a minute of breath-resolved measurements. In all presented cases we used mean values for analysis and only in case of any non-parametric distribution, medians were considered.

As every group mean value are contributed by each volunteer (of the corresponding group), the relative standard deviations (RSDs) in VOC abundances from each group were also calculated for each substance. The RSDs were calculated (in %) by rating sample standard deviations (SDs) over corresponding sample mean.

Statistically significant differences between age groups were assessed via repeated measurement ANOVA on ranks (Friedman repeated measures analysis of variance on ranks, Shapiro-Wilk test for normal distribution and post hoc Student–Newman–Keuls method for pairwise multiple comparisons between all groups; p value ≤ 0.005) in SigmaPlot software (version 14). In case of unequal in group size, one-way ANOVA was applied. All age groups were compared to each other.

For VOC abundances, from all pairwise comparisons, the differences in overall aging process is presented by referring to the corresponding values at the ‘Pre-teen’ group. Similarly, to compare the female sex-hormonal status, we selected those referring to the corresponding values at the ‘Adults on OCP’ group (i.e. representing an endocrine steady state due to daily oral contraception).

The volatomic similarities and differences among study groups are also assessed via principal component analysis (PCA) via the Unscrambler X software (version 10.3). In order to understand the correlations between differentially expressed VOCs (in different age groups) and their contributions to ageing, a dimension reduction factor analysis (Factor extraction via principal components method, factor scores via regression method and 1-tailed significance at p value ≤ 0.005) was performed in SPSS.