

KLK3 as a possible novel early biomarker of environmental exposure in young women living in Polluted area

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Research

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Abstract

Background: Kallikrein Related Peptidase 3 (KLK₃) is secreted by Skene's glands and, is considered ancestral homologues of the male prostate gland and has long been used as a biological marker of prostate cancer. Recent studies have shown that the synthesis of KLK₃ can be induced by steroid hormones in different tissues of women and in the menstrual cycle it seems to follow the cyclic variation of estradiol and progesterone. In addition, some environmental pollutants such as bisphenols, phthalate / DBP (DiButyl Phthalate) affect AR (Androgen Receptors) mediated signalling that directly regulates KLK₃ secretion. This suggests that environmental factors may play a role in KLK₃ secretion.

Methods: 61 healthy women living in a high environmental impact (HEI) area, 58 healthy women living in a low environmental impact (LEI) area were evaluated on possible presence or changes of KLK₃ in serum at different phases of the menstrual cycle: blood samples taken in the follicular phase 5th-6th day, ovulatory phase 12th -13th day and luteal phase 19th -20th day of the menstrual cycle. For this aim, an ultra-sensitive kit for KLK₃ with a detection limit of 0.001 ng / mL was used.

Results KLK₃ values showed two opposite peaks, women from HEI had a positive peak in the ovulatory phase with mean value of 9.90 ± 3.21 pg / mL while women from LEI had a negative peak in the ovulatory phase with mean values of 3.07 ± 1.49 pg / mL. Progesterone, showed a correlation with KLK₃. Women from HEI had higher KLK₃ values on average and no significant changes were evident between the three withdrawals in the different phases of the cycle. In contrast, women from LEI had a statistically significant decrease between the follicular and ovulatory phase ($p < 0.0001$) and a statistically significant increase ($p < 0.0001$) between the ovulatory and luteal phase.

Conclusions: The data obtained seem to go beyond the known role of KLK₃. The dosage of KLK₃ during the various phases of the menstrual cycle, simple to carry out and with low costs, can represent an effective and early biomarker to assess environmental exposure and useful to recognize the risk early and protect female health, not only reproductive.

Background

Kallikreins are a subgroup of serine proteases responsible for various physiological functions and, as enzymes, are capable of cleaving peptide bonds in proteins. In humans, plasma kallikrein (KLKB₁) has no known homologs, whereas tissue peptidases-related kallikrein (KLKs) constitute a family of 15 highly conserved serine proteases. These genes are localized to chromosome 19q13, forming the largest contiguous group of proteases in the human genome. The 15 members of the kallikrein-related serine peptidase (KLK) have different tissue-specific expression profiles. Initially, KLK₃ was believed to be absent from female tissues and fluids. However, KLK₃ has been detected in some female tissues (including breast, ovarian and endometrial tissues) and body fluids (serum, breast milk, amniotic fluid). The presence of KLK₃ in these female tissues appears to be closely associated with the regulation of steroid

hormones, particularly androgens and progestins. Estrogens themselves do not appear to affect KLK₃ regulation, but they may impair androgen-induced KLK₃ production. In tissues and fluids, KLK₃ is found in two molecular forms: free KLK₃ is the enzymatically active form and in a complexed form bound to protease inhibitors. Recent studies [1] suggest that KLK₃ can no longer be considered only as a specific prostate marker, but as a protein that could be produced by steroid hormone receptor cells under conditions of steroid hormone stimulation. Kallikreins are a rich source of disease biomarkers. The kallikrein locus is extraordinarily responsive to steroids and other hormones; in fact, at least 14 functional hormone response elements have been identified. A more complete understanding of the transcriptional regulation of kallikreins may help to formulate more concrete hypotheses about the physiological functions of kallikreins and their effectiveness as biomarkers. The best known of the kallikreins is KLK₃, commonly known clinically as Prostate-Specific Antigen, which is currently the serum biomarker for prostate cancer. The highest detectable proportion of the KLK₃ protein in serum is secreted by the prostate gland, and considerably higher concentrations, approximately 20,000 fold higher, can also be detected in human seminal plasma [2]. KLK₃ has been detected in salivary glands, brain, breast and other tissues, although at concentrations 100 times lower than the serum level [3]. Recent studies [4] report the presence of KLK₃ in female serum, in relation to PCOS [5] and hirsutism [6, 7]. Of particular interest is vaginal cervical fluid (CVF), which is a complex biological fluid that hydrates the mucosa of the lower female reproductive system. Extensive proteomic and biochemical studies on CVF have revealed that it contains large amounts of endogenous proteases and protease inhibitors, including an abundance of several members of the tissue kallikrein-related peptidase (KLK) family. The roles of KLKs in the lower female reproductive system are not fully understood, their activation is pH-dependent and there are also various other modes of regulation in the vagina. Roles have been postulated in physiological related to antimicrobial processes, vaginal and cervical epithelial desquamation, sperm transport and foetal membranes processing of, as observed in premature membranes rupture, through binding of known and unknown kallikrein substrates [8]. Despite their presence in human tissues and fluids, expression levels of KLKs vary widely, with highest expression observed in reproductive-related tissues and fluids. Zarghami and colleagues [9] report that KLK₃ is secreted cyclically during the menstrual cycle and appears to follow the peak concentration of Progesterone, with a delay of 10–12 days. Serum KLK₃ concentrations are highest during the mid-to-late follicular phase, fall continuously with a half-life of 3–5 days between the late follicular phase and mid-cycle and reach a minimum during the mid luteal phase. In many tissues, kallikrein expression is regulated by steroid hormones. In fact, the kallikrein locus is exceptionally responsive to hormones because each kallikrein is up-regulated by multiple hormones. For this reason, many researchers use kallikreins as a marker of hormone receptor activity [10, 11]. Progesterone regulates kallikrein expression in various tissues, sometimes through direct binding of the PR (Progesterone Receptor) to kallikrein promoters. KLK₂ and KLK₃ are both up-regulated by Progesterone in breast cancer cell lines [12]. KLK₃ is directly related to androgen and estrogen receptor up-regulation. Several data suggest that various kallikreins are regulated by steroid hormones, in particular androgens stimulate the KLK₃-secreting cell line while estrogens stimulate the KLK₁₀, KLK₁₁ and KLK₁₄-secreting cell line. Furthermore, KLK₃ expression is down-regulated by combined stimulation, confirming that estrogens

can antagonise and block androgen receptor activity [13]. Therefore, acquiring more detailed information about the mechanisms regulating kallikrein expression should be a priority and the kallikrein locus could be an important model in the era of genome-wide analyses. Lorenzetti and colleagues [14] highlight the ability of some environmental pollutants to stimulate prostatic epithelial cells in vitro, resulting in KLK₃ secretion. In addition, other chemicals such as bisphenols, phthalate / DBP (DiButyl Phthalate) affect AR (Androgen Receptors) mediated signalling that directly regulates KLK₃ secretion. This suggests that environmental factors may play a role in KLK₃ secretion [15]. In order to suggest a new bio-marker related to reproductive health effects caused by environmental factors, we will try to assess how the serum concentration of KLK₃ varies during the menstrual cycle in women from areas with different environmental impact.

Methods

119 women were enrolled in this study from September 2017 to April 2020 within EcoFoodFertility project (www.ecofoodfertility.it) a multidisciplinary research connecting human lifestyle and dietary habits to the environmental consequences of exposure to toxicants (approved by the Ethical Committee of the Local Health Authority Campania Sud-Salerno, Committee code 43/2015/06). 61 participants aged 22 to 37 (28.66 ± 4.43) years living in an area of the Campania Region (Southern Italy) with high environmental impact (HEI) (DL 136/2013-I 06/02/2014, ARPAC - *Regional Environmental Protection Agency of Campania*) the so-called “Land of fires” for the multiplicity of sources of pollution (illegal disposal of urban, toxic and industrial wastes, dumping practices, traffic, intensive agriculture), 58 participants aged 23 to 34 (27.3 ± 3.18) years living in an area of the same region but with low environmental impact (LEI) (Fig. 1).

Figure 1: Map of Campania region (Southern Italy) with the two areas. Circled in red high environmental impact (HEI) area, so-called “land of fires”, circled in green low environmental impact (LEI) area. ARPAC Annuario dei Dati Ambientali in Campania 2006. [(accessed on 12 June 2015)]; Available online: <http://www.arpacampania.it/documents/30626/51722/Siti+Contaminanti.pdf>.

Participants in the study were selected by examining the quality and quantity of environmental pollution in the area in which they lived, and they were all of childbearing age. A specific “participant form” was proposed, in which anamnestic and clinical data are reported in order to create a database. Participants reported symptoms and signs of any medical conditions, hirsutism, acne and anamnestic data related to their lifestyle, use and/or abuse of alcohol, smoking and possible drug use. Participants had no major chronic diseases and had resided permanently in the selected areas for at least 5 years, were not professionally exposed to risk factors and had not taken oral contraceptive pills for at least 2 years. They denied having used drugs in the 12 months before the blood draw. Participants were asked about their age at menarche, whether they were nulliparous/multiparous, and whether they had experienced spontaneous/voluntary abortions. The participants had normal menstruation, the duration of the whole cycle varied from 28 to 30 days; and regularity, volume and duration were regular. In addition, BMI (body

mass index), waist circumference, waist-to-hip ratio and Ferriman-Gallwey score for hirsutism were calculated.

Blood samples were collected during the 3 phases of the menstrual cycle, namely on days 5–6 (follicular phase), 12–13 (ovulatory phase) and 19–20 (lutein phase). KLK₃ was assayed in all three sera sample and progesterone was assayed on the third sample.

The method we have chosen for the KLK₃ assay in serum is the Access Hybritech KLK₃ assay, developed by Beckman Coulter, which is a two-site immunoenzymatic assay, also known as sandwich immunoassay, using the principle of chemiluminescence and producing light directly proportional to the concentration of KLK₃ in the sample. We used the UniCel Dxl 600 Access Immunoassay System. The whole database, covering all participants, was subjected to a statistical analysis for each area and for the entire enrolled population. Assuming a significant difference between the groups belonging to the two selected areas, differences were found between the 61 participants from the high environmental impact area and the 58 participants from the low environmental impact area. A statistical analysis for each area and for the whole enrolled population was performed across the two groups using the Student's "t"-Test. Signed informed consent was received from all participants in this study, in accordance with the ethical principles of experimentation (institutional or regional) and the Declaration of Helsinki of 1975, revised in 2000.

Results

Table 2 shows the variables of the two groups under examination; data were obtained from the forms completed by the participants and from the clinical evaluation performed by our collaborators.

Table 2
population studies

Participants	High environmental impact (n = 61)	Low environmental impact (n = 58)	P-Value
Age (years old \pm SD)	28.66 \pm 4.43	27.3 \pm 3.18	N.S.
Smokers	8.19 %	5.17 %	N.S.
Alcohol	9.84 %	1.72 %	p < 0.05
Drugs	0.0 %	0,0 %	-
Age at menarche (years old)	11.- 13	11.- 12	N.S.
Nulliparous	86.9 %	79.3 %	N.S.
Multiparous	13.1 %	20.7 %	N.S.
Previous abortions	14.8 %	3.4 %	p < 0.05
BMI score	24.2–29.4	23.1–27.4	N.S.
Waist circumference (cm)	72–110	69–90	N.S.
Waist hips	0.81–0.95	0.55–0.78	p < 0.05
Ferriman – Gallwey:			
Score 1	75.4 %	75.9 %	N.S.
Score 2	24.6 %	24.1 %	N.S.

KLK concentrations in the serum of the young women showed an irregular behaviour. Indeed, in 14 out of 58 participants belonging to the low-impact group (24.1%), and in 10 out of 61 participants belonging to the high-impact group (16.4%), KLK₃ was not detectable in any of the three samples per menstrual cycle with the method we used (detection limit 0.001 ng/mL). Therefore, only 95 out of all participants (79.8 %) were included in the further statistical analysis and comparative evaluation. The BMI of the two groups was homogeneous, there were only two categories: normal and overweight. Comparing the two groups with the KLK₃ values, no significant changes were observed and the trend during the three phases of the cycle was superimposable. Comparing KLK₃ values with Ferriman & Gallwey scores (scores 1 and 2), no significant variations were observed and the trend during the cycle was superimposable. The comparison of KLK₃ and Progesterone values performed in the luteal phase, showed a significant correlation (p < 0.001). In fact, the mean value of progesterone in the high environmental impact group was 6.67 \pm 5.73 ng/mL, while in the low environmental impact group it was 14.1 \pm 6.19 ng/mL. We noted that the

progesterone limit value of 14.1 ng/mL corresponded to an opposite trend in KLK₃ concentrations. For this value, 90.3% of the participants belonging to the high environmental impact group had a positive KLK₃ peak in the ovulatory phase and 93.6% of the participants belonging to the low environmental impact group had a negative peak in the ovulatory phase (Fig. 2).

Figure 2: 90.3 % of participants living in area with high environmental impact.

93.6 % of participants living in area with low environmental impact.

Comparing KLK₃ values with the parity of participants, the following data emerged: in the nulliparous women, the value of the first sample (follicular phase: 3.52 ± 1.3) increased significantly in the second sample (ovulatory phase: 15.82 ± 3.3) and then decreased in the third sample (luteal phase: 6.43 ± 2.7). In multiparous women, the value in the first sample (follicular phase: 9.96 ± 2.5) decreased in the second sample (ovulatory phase: 5.71 ± 2.5) and then increased in the third sample (luteal phase: 7.99 ± 3.4) (Figure 3). KLK₃ values in the high-impact group was on average higher than those in the low-impact group during all three phases of the menstrual cycle. In the high impact group, changes in KLK₃ concentrations across the three phases of the menstrual cycle (8.95 ± 4.43 : 9.90 ± 3.21 : 9.63 ± 2.87) showed no significant changes, while they showed significant changes ($p < 0.001$) between follicular phase (7.18 ± 1.57) and ovulatory phase (3.07 ± 1.49) same statistical significance between ovulatory phase (3.07 ± 1.49) and lutein phase (6.29 ± 1.97) in the low impact group.

Discussion

Female participants living in low environmental impact area

Female study participants were aged from 23 to 34 (27.3 ± 3.18). Out of a total of 58 female participants included in the study, 75.9 % had detectable KLK₃ (values greater than 0.001 ng/mL). Previous studies reported detectable KLK₃ in 50% of non-pregnant women [17–19]. Filella and colleagues [19] in 1996 detected the presence of KLK₃ in 58% of female sera, Escobar-Morreale and colleagues [20] in 1998, detected the presence of 36.4 % KLK₃ in the serum of women with normal menstrual cycles and Mannello and colleagues [21] in 2001, did not detect the presence of KLK₃ in female serum. Normal serum levels of KLK₃ in women range from 0.02–0.06 ng/mL [22]. In this group, the highest concentrations of KLK₃ were found during days 5–6 of the menstrual cycle (7.18 ± 1.57 pg/mL), a second, smaller peak was observed on days 19–20 (6.29 ± 1.97 pg / mL). Yu and colleagues found low levels of KLK₃ during days 10–23 and a peak at the end of the cycle or at the beginning of the next cycle [23]. For this group, the peak was also seen at the beginning and at the end of the cycle in our study.

Changes in KLK₃ values were statistically significant: comparison of values between the follicular phase (7.18 ± 1.57 pg/mL) and the ovulatory phase (3.07 ± 1.49 pg/mL) showed a significant decrease with $p < 0.0001$; comparison of values between the ovulatory phase (3.07 ± 1.49 pg/mL) and the lutein phase (6.29 ± 1.97 pg/mL) showed a significant increase with $p < 0.0001$ (Fig. 4).

Black and colleagues in 1972 [24], found mean Progesterone levels of 1.8 ± 1.1 pg/mL (days 0–11 of the cycle); 3.1 ± 1.8 (days 12–14 of the cycle); 12.1 ± 7.8 pg/mL (days 15–25 of the cycle) and 4.3 ± 3.3 pg/mL (days 26–28 of the cycle). In our study, progesterone values were measured on days 19–20 of the cycle with values ranging from 0.5 to 28.6 pg/mL (14.1 ± 6.19 pg/mL).

Female participants living in high environmental impact area

Female study participants were aged from 22 to 37 (28.66 ± 4.43). Out of a total of 61 female participants included in the study, 83.6 % had detectable KLK₃. In this group, the highest concentrations of KLK₃ were found on days 12–13 of the menstrual cycle (9.90 ± 3.21 pg/mL). Changes in KLK₃ values in the three cycle phases: follicular phase 8.95 ± 2.90 , ovulatory phase 9.90 ± 3.21 and lutein phase 9.63 ± 2.87 , were not statistically significant (Fig. 5).

In this group, progesterone values were detected on days 19–20 of the cycle with values ranging from 0.4 to 21.2 ng/mL (6.67 ± 5.72 ng/ml). We found statistically significant ($p < 0.0001$) changes in progesterone values among the two groups studied, depending on their residence (Fig. 6).

Figure 6: mean and standard deviations of progesterone values in the two groups

Progesterone is the main endocrine product of the corpus luteum during the menstrual cycle [9]. In vitro studies have shown that sera obtained during the menstrual cycle can stimulate KLK₃ production in a breast cancer cell line (T-Line 47D). Measurement of KLK₃ protein and KLK₃ mRNA levels showed that the ability of serum to induce KLK₃ production in cancer cells was proportional to serum progesterone levels, with the greatest stimulation occurring with serum containing more progesterone (days 22–24 of the menstrual cycle). With luteal phase serum, KLK₃ mRNA expression was significantly increased [9].

In 79.8% of all participants, KLK₃ is detectable in serum and is expressed in the three phases of the menstrual cycle: in the follicular phase the minimum value is 3.52 pg / mL and the maximum 17.74 pg / mL, in the ovulatory phase the minimum value is 0.11 pg / mL and the maximum value is 20.36 pg / mL and in the luteal phase the minimum value is 1.12 pg / mL and the maximum value is 19.24 pg / mL. The concentration of KLK₃ in the blood serum of the young nulliparous women who participated in this work showed a negative peak in the ovulatory phase in 46.3% and a positive peak in the ovulatory phase in 53.7%. On the other hand, in the multiparous it showed a negative peak in ovulatory phase in 86.3%. As previously reported, comparison of KLK₃ values with the anamnestic data showed a significant change ($p < 0.05$) between the two groups of participants for alcohol use and previous abortions, semeiotic evaluations between the two groups showed a significant change ($p < 0.05$) for the waist / hip ratio. Changes in KLK₃ concentrations have been reported in several studies reflecting serum progesterone levels, albeit with a delay of 12 to 16 days. In a biphasic profile, KLK₃ is produced in target tissues in response to increased progesterone levels in the ovulatory phase, KLK₃ diffuses into the blood and is detected in the luteal phase. Progesterone spikes during the luteal phase are able to provide a spike in

KLK₃ in the first follicular phase of the next cycle [4, 9]. The interpretation of the negative and positive peaks of KLK₃ in serum found in the serum of participants from LEI area and HEI area respectively is different, perhaps some mechanism of KLK₃ secretion is independent of progesterone. In the HEI area participants were found to be higher on average with insignificant variation between the three phases of the cycle, it is conceivable that other stimuli, such as environmental factors, prompt a continuous secretion by the epithelial cells of the glands by Skene. Indeed, in other studies it has been shown that prostate epithelial cells respond to the stimulation of certain pollutants [14]. The concentrations of KLK₃ in the blood serum of participants living in LEI area showed a negative peak in the ovulatory phase with significant changes ($p < 0.0001$) between the follicular and ovulatory phase and the ovulatory and luteal phase, this cyclicity seems to respond to endogenous stimuli.

Furthermore, comparing the data of the two groups, we noted a significantly different ($p < 0.001$) dosage rate in the samples of the three cycle phases, 75.9% for the LEI zone and 83.6% for the HEI zone, probably the result of increased stimulation that may occur in the HEI zone. The roles of KLK₃ in the female reproductive system are not fully known. The activation of KLK3 in vaginal cervical fluid (CVF) is dependent on vaginal pH, but there are other mechanisms that regulate KLK₃ in the vagina. It has been hypothesized that KLK₃ plays a role in physiological functions related to antimicrobial processes, vaginal and cervical epithelium desquamation, spermatozoa transport and movement and more recent works indicate a probable role as an immunoregulator.

Conclusions

This study highlights an "interference" in the secretion of KLK₃, in women who permanently reside in HEI area. Environmental pollutants in the HEI area may exert an endocrine interference that stimulates the Skene's glands, in fact during the menstrual cycle the concentrations of KLK₃ are higher and with minimal fluctuations. A greater understanding of the functional roles of KLK₃ in the female reproductive system could lead to new diagnostic and therapeutic modalities for conditions such as vaginal infections, vaginal atrophy, and a probable role on sperm and endometrium.

This pilot study suggest a new role of KLK₃ in women. Its changes during the phases of the menstrual cycle demonstrate an action different other than simple secretion. The data obtained seem to go beyond the functions advanced by other authors on its role in antimicrobial processes, vaginal and cervical epithelial desquamation, and spermatozoa transport. The correlation with progesterone in the different phases of the menstrual cycle of young women and the different peaks in the ovulatory phase in the two study groups for areas with different environmental impact, allows us to consider the cyclic secretion and the concentration of KLK₃ as a new marker of environmental exposure in women. However, further studies, larger numbers and more homogeneous age group sampling will be needed to better understand the functions of KLK₃ in a woman's serum on the different days of the cycle.

Declarations

Acknowledgements

Not applicable

Authors' contributions.

Raimondo Salvatore, Gentile Mariacira Ferrara Ida, Montano Luigi: Drafted manuscript. Montano Luigi, Piscopo Marina: Edited and revised manuscript. Gentile Tommaso, Gentile Mariacira and Raimondo Salvatore: Analyzed data. Ferrara Ida, Crescenzo Claudia and Palmieri Mariangela: Collected the data. Cuomo Felice, Gentile Mariacira, Esposito Giusy and De Filippo Stefania: performed experiments. Raimondo Salvatore and Montano Luigi: Conceived and design research.

All authors read and approved the final manuscript.

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Availability of data and materials

Not applicable.

Declarations

Ethics approval and consent to participate

Ethical Committee of the Local Health Authority Campania Sud-Salerno, approved EcoFoodFertility project (Committee code 43/2015/06). All participants had provided informed consent before participation.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Table 1

Table 1 is available as a download in the Supplementary Files section.

Figures

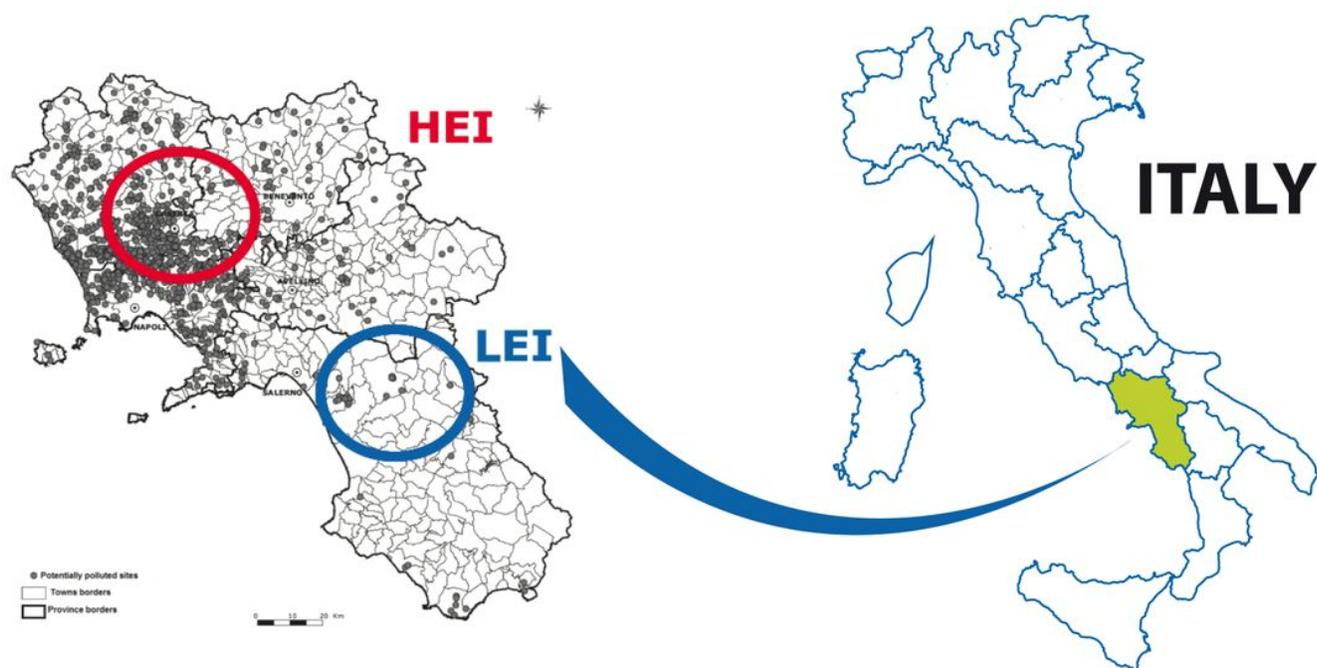


Figure 1

Map of Campania region (Southern Italy) with the two areas. Circled in red high environmental impact (HEI) area, so-called “land of fires”, circled in green low environmental impact (LEI) area. ARPAC Annuario dei Dati Ambientali in Campania 2006. [(accessed on 12 June 2015)]; Available online: <http://www.arpacampania.it/documents/30626/51722/Siti+Contaminanti.pdf>. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

Comparison between KLK₃ and Progesterone

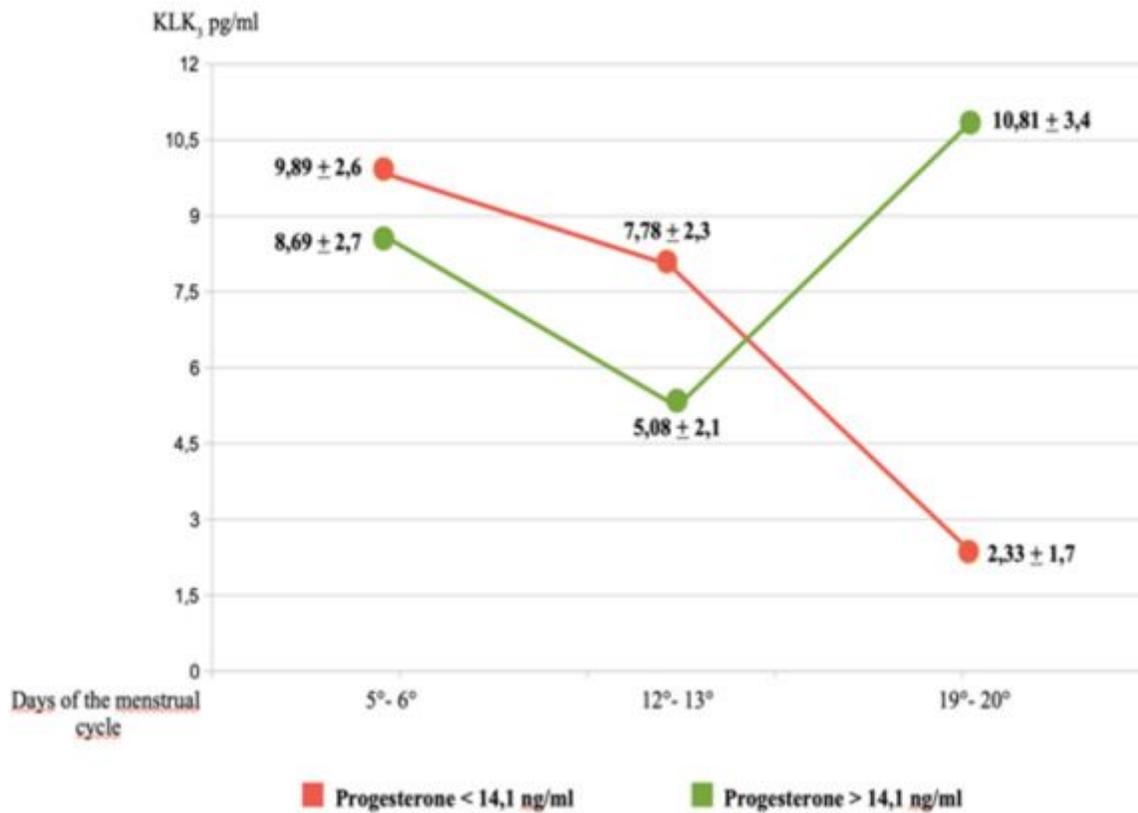


Figure 2

90.3 % of participants living in area with high environmental impact. 93.6 % of participants living in area with low environmental impact.

Comparison between KLK₃ and Nulliparous / Multiparous

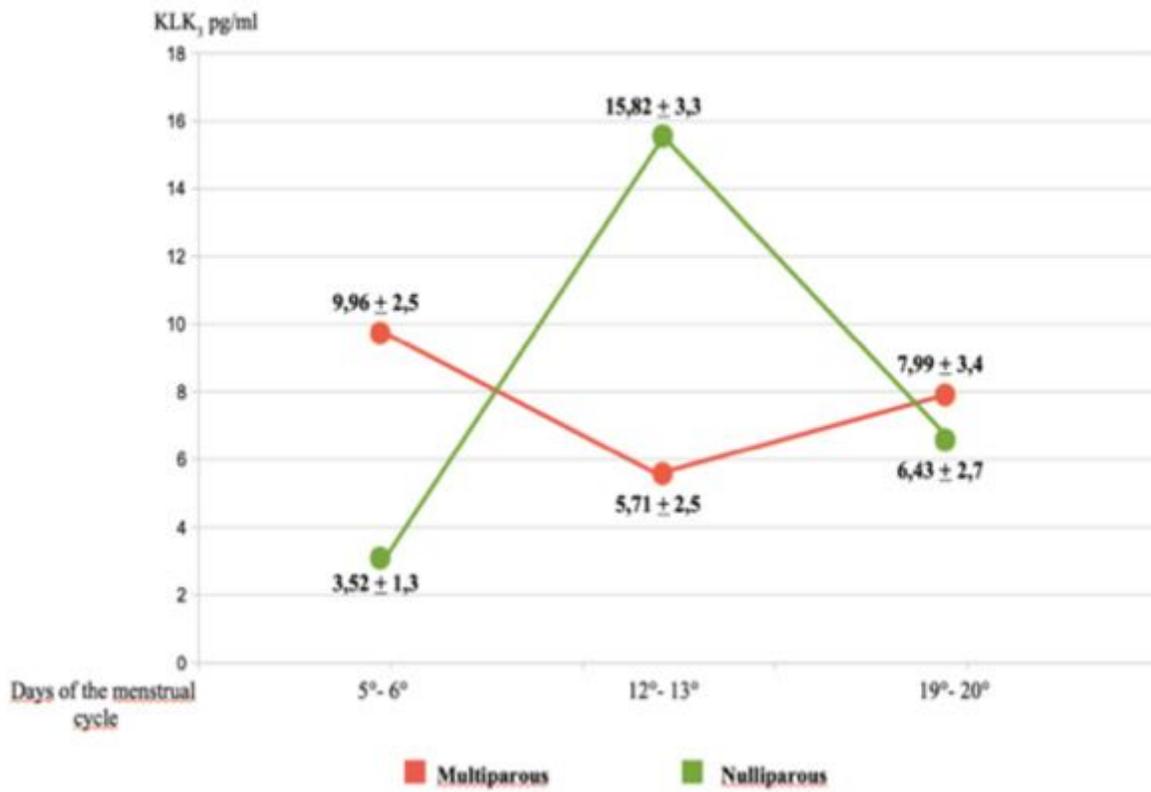


Figure 3

Comparison between KLK₃ and Nulliparous and Multiparous

KLK₃ values in the Low Environmental Impact group

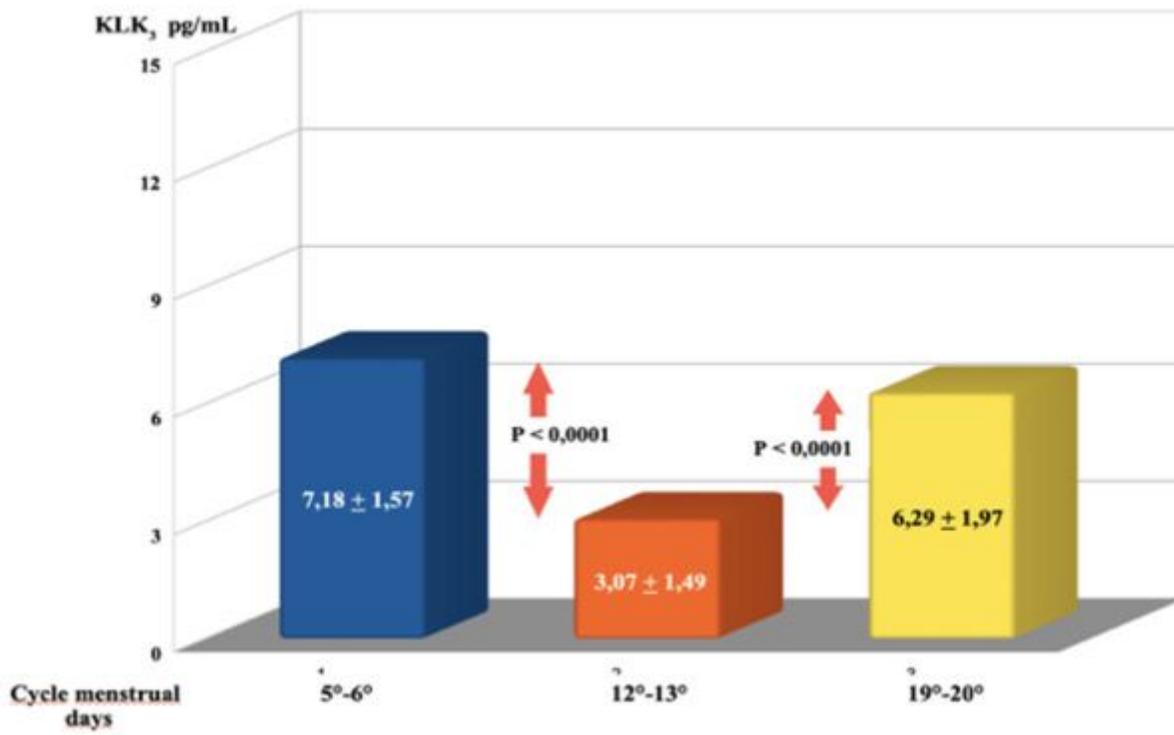


Figure 4

KLK3 values in the Low Environmental Impact (LEI) group

KLK₃ values in the High Environmental Impact group

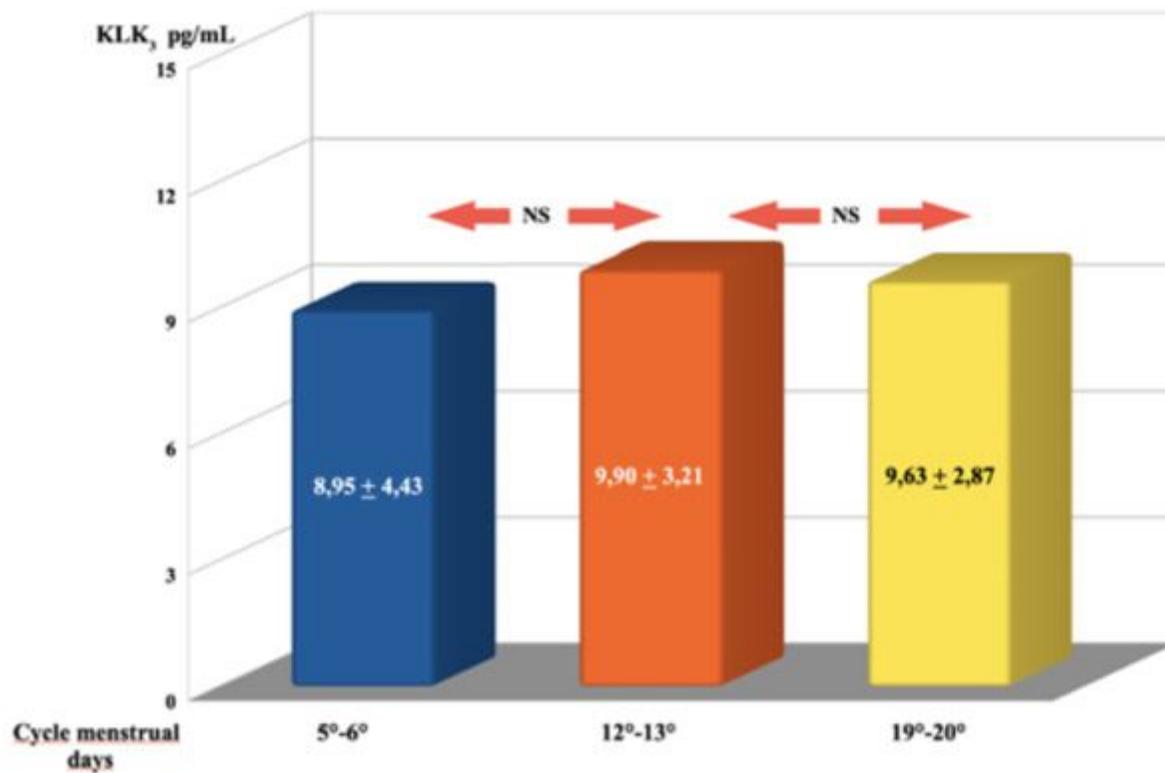


Figure 5

KLK₃ values in the High Environmental Impact (HEI) group

Mean and standard deviations of Progesterone values in the two groups

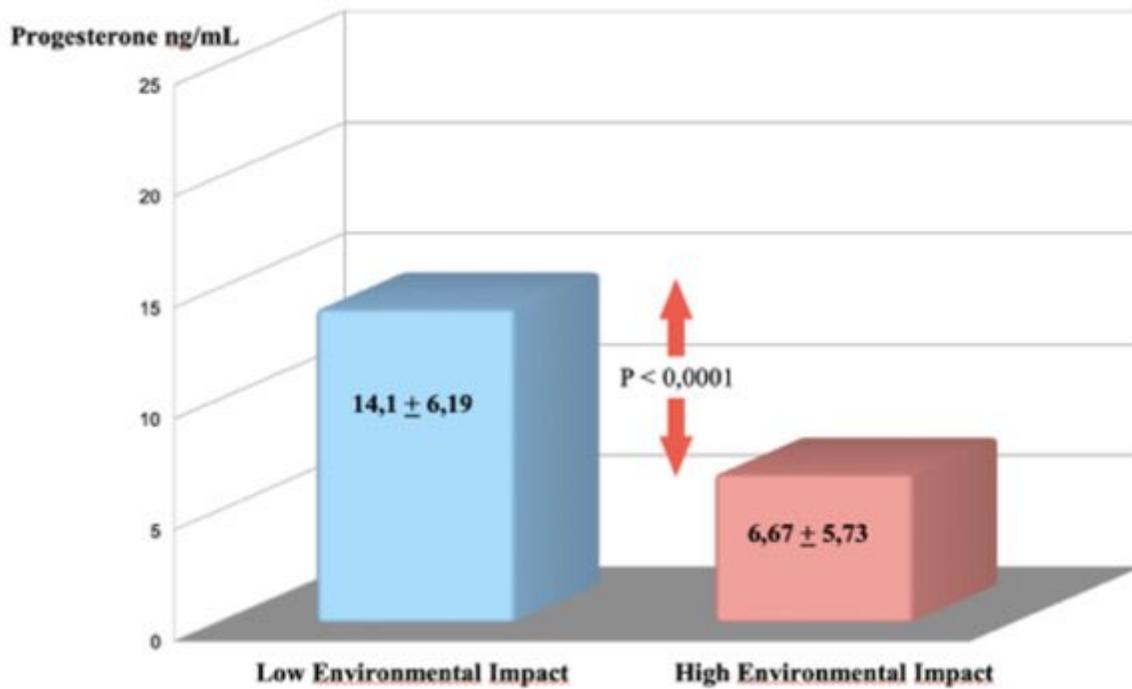


Figure 6

mean and standard deviations of progesterone values in the two groups

Supplementary Files

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- [Onlinetable1.png](#)