Volatile Signals during Pregnancy: A Possible Chemical Basis for Mother-Infant Recognition

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Abstract Human pheromones have a role in regulating relationships, and apparently influence partner choice and mother-infant recognition. We analyzed the chemical content of volatiles from sweat patch samples from the para-axillary and nipple-areola regions of women during pregnancy and after childbirth. Solid phase microextraction (SPME) was used to extract the volatile compounds, which were then characterized and quantified by gas chromatography-mass spectrometry (GC-MS). During pregnancy, women developed a distinctive pattern of five volatile compounds common to the para-axillary and nipple-areola regions (1-dodecanol, 1-1′-oxybis octane, isocurcumenol, α-hexyl-cinnamic aldehyde, and isopropyl myristate). These compounds were absent outside pregnancy and had slightly different patterns in samples from the two body areas. Differentiation of the volatile patterns among pregnant women may help newborns to distinguish their own mothers.

Keywords Gas chromatography-mass spectrometry (GC-MS) · Human pheromones · Solid phase microextraction (SPME) · Sweat patches · Volatile compounds

Introduction

In animal species, recognition between individuals is a prelude to any kind of further interaction. Recognition between mother and newborn is a fundamental behavioral interaction that is worthy of systematic investigation. The emotional relationship between a mother and her newborn begins with mutual recognition, which starts during gestation and continues through and after birth, augmented by body contact and lactation. Imprinting takes place through visual, auditory, and olfactory learning, which occurs very early during the so-called "critical period" (Chiarelli, 2003). Consequently, from the beginning of pregnancy, olfaction seems to represent an Ariadne's thread that permits the infant after birth to find its mother.

Pheromones regulate reproductive behavior in many mammalian species. Once released in the environment, through urine or glandular secretions, these volatiles reach other individuals, signaling mating availability and strengthening ties between mother and offspring, as well as regulating social relationships (Scalfari, 1994; Chiarelli, 2001). In non-human vertebrates, pheromones are detected by a specific sensory apparatus, the vomeronasal system, composed of a peripheral organ located at the base of the nasal septum, the vomeronasal nerve, and a nerve center, the accessory olfactory bulb. The vomeronasal system is separated and independent from the main olfactory system (Døving and Trotier, 1998; Chiarelli, 2003). It is triggered by a different class of volatiles called "odorants," and is present in many reptiles and in almost all mammals, but it is absent in fish and birds, even if they possess a main olfactory system.

Primates, long considered functionally non-microsomatic, were thought previously to show complex olfactory communication only in prosimians and in some New World monkeys. Now it is known that even higher primates use pheromones to recognize conspecific individuals and to mark territory (Michael and Keverne, 1968; Michael et al., 1971; Keverne, 1983). Furthermore, protein-pheromone complexes present in the secretions released by scent-marking of some non-human

primates have been shown to activate vomeronasal receptors, particularly for sexually related behaviors and intraspecific identification of individuals (Smith et al., 2001; Hayes et al., 2004; Palagi et al., 2004; Vaglio et al., 2004).

Formerly, it was widely held that the human vomeronasal organ was vestigial, and even the existence of pheromonal communication in humans was contested. Support for a role of pheromones in human behavior came from observational studies, e.g., synchronization of the menstrual cycle (McClintock, 1971), but this role was often denied because there seemed to be an insufficient neuro-anatomic basis for such complex behavior (Pelucchi, 2006).

Recently, a new class of olfactory receptors (trace amino-associated receptors, TAAR) was discovered in the olfactory epithelium of mice (Liberles and Buck, 2006). Genes similar to those responsible for the control of these receptors in mice have been identified in humans and fish, which suggests evolutionary conservation and lends support to the hypothesis that the human pheromonal response is mediated by receptors located in the main olfactory system (Pelucchi, 2006).

Putative human pheromones are steroids present in the secretions of exocrine glands (Taylor, 1994; Stern and McClintock, 1998; Grosser et al., 2000; Pause, 2004; Grammer et al., 2005; Hauser et al., 2005). Estrogen derivatives are present in females (the so-called copulins - mixtures of aliphatic acids such as acetic, propionic, butyric, isovaleric, and isocaproic acid with estratetraenol), and androgen derivatives are present in males (androstenol, androstenone, and androstadienone). Studies concerning the most volatile compounds of human sweat (Zeng et al., 1991 1996a, b; Bernier et al., 2000; Curran et al., 2005) have shown that the characteristic odor produced by the para-axillary region is due to the presence of volatile C6-C11 acids; the most abundant is *E*-3-methyl-2-hexenoic acid (*E*-3M2H).

Human pheromones also may play a role in offspring identification. Odor cues from newborns are salient to their mothers (Kaitz et al., 1987). Mothers can distinguish the odor of their own newborn baby from that of other newborns (Schaal et al., 1980; Kaitz et al., 1987; Chiarelli,

2001). Experiments also have demonstrated that adults can recognize gender and individuality of non-related children (Ligabue Stricker, 1991; Chiarelli, 2001). Thus, body odors can provide humans with information about the individual identity of their offspring (Doty, 1981; Porter and Moore, 1981; Porter et al., 1983, 1985; Russell et al., 1983; Curran et al., 2005; Olsson et al., 2006).

Children usually prefer parts of clothes that have been in contact with the axilla and worn by their own mothers to clothes worn by other mothers (Schaal et al., 1980). Thus, pheromones seem to have a fundamental role in the mechanism of mother-child identification (Porter et al., 1983; Porter and Winberg, 1999). Breast-fed versus bottle-fed infants show different reactions to maternal odors. Breast-fed infants are exposed to salient maternal odors and rapidly become familiarized with their mother's unique olfactory signature (Cernoch and Porter, 1985). Apparently, orientation to lactating-breast odors is an inborn adaptive response of a newborn (Porter et al., 1991).

Without doubt, naturally occurring odors play a role in mediating infant behavior. Even fetal olfactory learning seems to occur (Varendi et al., 1996), and breast odors from the mother exert a pheromone-like effect at the newborn's first attempt to locate the nipple. Newborns generally are responsive to breast odors produced by lactating women (Porter and Winberg, 1999). Olfactory recognition may be implicated in the early stages of the mother-infant attachment process, when newborns learn to recognize the own mother's unique odor signature: this process is facilitated possibly by high norepinephrine release and the arousal of the *locus coeruleus* at birth (Winberg and Porter, 1998). Human infants are responsive to maternal odors that begin shortly after birth. They show an attraction to amniotic fluid (AF) odor that may reflect fetal exposure to that substance (i.e., prenatal olfactory learning) (Varendi et al., 1996). Moreover, human AF seems to carry individualized odor properties, which are hypothesized to initiate parent-infant interactions (Schaal and Marlier, 1998).

On the basis of these literature findings, we hypothesized that women probably develop a volatile profile through pregnancy and childbirth that enables identification of the mother by the

newborn. The aim of the present research was to understand how the volatile pattern of pregnant women changes during pregnancy and, consequently, provide the possible chemical basis for mother-infant olfactory recognition.

Methods and Materials

Sampling Procedures Sweat samples were collected from a group (*N*=20) of 30 to 40 yr-old pregnant women (mean age=36.8 yr). Sweat patches (PharmChem Inc., Fort Worth, TX, USA) were applied to para-axillary and nipple-areola regions for at least 24 hr. The patch is basically a large band aid with an untreated cellulose pad that is sterile and devoid of chemical additives. Blank patches were prepared under the same experimental conditions and used as controls. Isopropanol was used to clean the skin prior to application of the patch. The patches can be worn for up to 7 d, with a minimum of 24 hr, whereas patch "overlays," included in the kit, can be placed over the original to provide even more adhesion.

Samples were collected between July 2006 and October 2007 from three periods during pregnancy and after childbirth to permit an evaluation of the volatile pattern during and after the gestational period: 1. The second month of gestation (7-10th week of gestation); 2. The last month of gestation (at about the 35th week of gestation); and 3. After childbirth (at least 6 mo after birth).

These particular three time periods were selected on the basis of pragmatic criteria: 1) the earliest possible time period during pregnancy (based on first awareness of pregnancy); 2) the latest possible time period during pregnancy (based on avoiding the risk of premature births); and 3) a distant time period after pregnancy (based on the need for the subject women to begin their regular rhythm of life and, above all, their menstrual cycles).

All participants in the study signed an informed consent form for this totally non-invasive investigation. Italian protocols for human subjects were followed. The participants also responded

to a short questionnaire. On the first day, a brief personal obstetrician anamnesis (personal history) was developed, and on the second, questions were asked regarding potential food and/or emotional anomalies (e.g., stressful occurrences, sexual activity) encountered during the period of sweat patch application that could potentially modify the pheromonal profile..

Sample collection was carried out from the para-axillary and nipple-areola regions with the following protocol: 1. Banned fragrances and deodorants were not used in the previous 24 hr; 2. Unusual food and/or stresses and emotional behaviors were not present in subjects; 3. Sweat patches were applied, one to the para-axillary and one to the nipple-areola region; 4. Sweat patches were removed after at least 24 hr and inserted into 20 ml solid phase microextraction (SPME) vials, which were hermetically closed by teflon-faced rubber septa and aluminium seals with a fit hand crimper; 5. Vials with the sweat patches were maintained at -20°C and were transported to the laboratory for analysis.

Extraction, Characterization, and Quantification of Volatile Compounds A SPME syringe needle, coated with 30 μm of polydimethylsiloxane, was introduced through the vial septum (Supelco, Bellefonte, PA, USA). The vial was held at 60°C, and the fiber was exposed to the headspace above the sample for 20 min. Adsorbed volatile analytes were analyzed by gas chromatography-mass spectrometry (GC-MS) by using a DSQ mass spectrometer (Thermo Electron Corporation, Waltham, MA, USA) operated in EI (70 eV) mode and directly coupled to a Focus gas chromatograph (Thermo Electron Corporation, Waltham, MA, USA). The GC was equipped with a fused silica HP 5-MS capillary column (30 m x 0.25 mm crossbonded 5%-phenyl-95%-dimethylpolysiloxane, film thickness 0.50 μm, Agilent Technologies, Santa Clara, CA, USA). The injector and transfer line temperatures were maintained at 220°C and 250°C, respectively. Injections were made in splitless mode with a constant flow of helium carrier gas of 1.1 ml/min. The oven temperature program, started at 40°C, was held for 3 min and then raised by 10°C/min to 100°C, and in a second step, by 5°C/min to the final temperature of 250°C. The eluted compounds

were tentatively identified by comparing the experimental spectra with those of the NIST mass spectral library, Version 2.0 (Thermo Electron Corporation, Waltham, MA, USA). Authentic standards for confirmation of compound identities were purchased from Sigma Aldrich, Milan, Italy. The relative amounts of the compounds of interest were determined by integrating the areas of the corresponding peaks in the Total Ion Current (TIC) profile. Percentages were calculated with respect to the most abundant compound.

Statistical Analyses The peaks in the Total Ion Current (TIC) profile were taken as measures of the relative amounts of the volatile compounds of interest. Each compound was considered as a variable and, for further analysis, we assigned each compound a categorical value of 0 or 1: 1 indicated the presence of compound and 0 its absence. Questionnaires provided information about lactation, menstrual cycle, and the contingent occurrence of food and/or emotional anomalies.

The goal of the statistical analysis was to determine whether the presence/absence of the five volatile compounds was influenced by body area (A = para-axillary region, B = nipple-areola region); sampling time (1 = beginning of gestation, 2 = end of gestation, or 3 = after childbirth); and other variables collected on the questionnaire as listed above. To study these possible associations, we used a Multiple Logistic Regression model, a particular type of generalized linear model for binary data that is used to study the relationship among dependent variables (also known as response and independent binary variables) (statistical package "R", Version 2.7.2, Free Software). The dependent variable was the presence/absence of each volatile compound (1-dodecanol, 1-1´-oxybis octane, isocurcumenol, α-hexyl-cinnamic aldehyde, or isopropyl myristate, see below), whereas the independent variables were the body area, the sampling time, the contingent occurrence of food and/or emotional anomalies, and the possible presence of lactation and/or menstrual cycle.

A re-weighted least squares (IWLS) method (statistical package "R", Version 2.7.2, Free Software) was used to fit the data in the regression model. Statistical significance of the associations

was determined by analysis of variance (ANOVA) ($\alpha = 0.1$ for significance) (statistical package "R", Version 2.7.2, Free Software).

Results

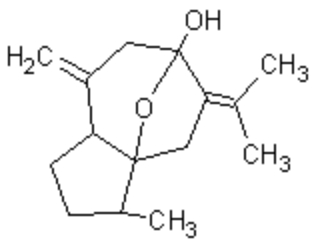
Identification of Volatile Compounds During pregnancy all women developed a distinctive volatile pattern represented by five compounds common to the para-axillary and nipple-areola regions (Fig. 1). The five compounds (R_t) are: (**A**) 1-dodecanol (17.83 min); (**B**) 1-1′-oxybis octane (21.60 min); (**C**) isocurcumenol (21.79 min); (**D**) α-hexyl-cinnamic aldehyde (23.27 min); and (**E**) isopropyl myristate (24.54 min). Identification of all commercially available compounds (**A**, **B**, **D**, **E**) was confirmed by comparison of the retention times and mass spectra with those of authentic standards. Compound **C** was identified as isocurcumenol by comparison with the EI mass spectrum of isocurcumenol reported in the literature (Yang et al., 2005), where the mass spectrum of the isomer curcumenol also is reported: the fragmentation of the two isomers is completely different.

$CH_3(CH_2)_{11}OH$ (**A**)

$[CH_3(CH_2)_7]_2O$ (**B**)

$C_6H_5CH{=}C[(CH_2)_5CH_3]CHO$ (**D**)

$CH_3(CH_2)_{12}COOCH(CH_3)_2$ (**E**)



(**C**)

The five compounds, which were absent in the analysis of blank patches, were the only recurrent volatile compounds within the three sample periods. They were not detected in an equivalent control sample of non-pregnant and non-lactating females (Fig. 1). Thus, the volatile composition changes during pregnancy.

Effect of Body Area, Time, Lactation, Menstrual Cycle, Food and/or Emotional Anomalies The effects of various independent variables from the Multiple Logistic Regression on the dependent variables (presence/absence of 1-dodecanol, 1-1´-oxybis octane, isocurcumenol, α-hexyl-cinnamic aldehyde, or isopropyl myristate) are as follows:

1. the occurrence of lactation during the third sampling period (after childbirth) did not affect the presence/absence of any volatile compound (1-dodecanol: $P = 1$; 1-1´-oxybis octane: $P = 0.400$; isocurcumenol: $P = 0.209$; α-hexyl-cinnamic aldehyde: $P = 0.270$; and isopropyl myristate: $P = 1$), nor did the return of the menstrual cycle (1-dodecanol: $P = 0.209$; 1-1´-oxybis octane: $P = 0.400$; isocurcumenol: $P = 0.209$; α-hexyl-cinnamic aldehyde: $P = 0.270$; and isopropyl myristate: $P = 1$).
2. no independent variable (body area, sampling time, food and/or emotional anomalies) affected the presence of α-hexyl-cinnamic aldehyde (model with food anomalies as independent variable – body area: $P = 0.142$; second sample period: $P = 0.998$; third sample period: $P = 0.788$; food: $P = 0.469$; model with emotional anomalies as independent variable – body area: $P = 1$; second sample period: $P = 0.998$; third sample period: $P = 0.584$; emotions: $P = 0.436$).
3. no independent variable (body area, sampling time, food and/or emotional anomalies) affected the presence of isopropyl myristate (model with food anomalies as independent variable – body area: $P = 1$; 2nd sample period: $P = 1$; third sample period: $P = 1$; food: $P = 1$; model with emotional anomalies as independent variable – body area: $P = 1$; second sample period: $P = 1$; third sample period: $P = 1$; emotions: $P = 1$);
4. the combination of body area with food anomalies ($P = 0.066$) and emotional anomalies ($P = 0.046$) affected the presence of isocurcumenol;
5. the combination of sampling time with food anomalies ($P = 0.051$) and emotional anomalies ($P = 0.073$) affected the presence of 1-1´-oxybis octane;

6. the combination of sampling time and emotional anomalies (P = 0.053) affected the presence of 1-dodecanol.

Additionally, a *Chi-square* test supports these conclusions (data not shown). Since the control values were equal to 0, they were not included in the statistical analysis.

Qualitative Analyses The patterns of the relative occurrence of the five volatile compounds in the samples varied with the body regions and the time of sampling (Fig. 2). In early gestation nearly all samples from the nipple-areola region contained all five volatile compounds. Fewer samples from the para-axillary region had the compounds during early gestation. In late gestation the five components were detected in nearly all samples from both body areas, whereas after childbirth the fraction of samples that contained the compounds declined dramatically in both body areas. After childbirth, 1-1′-oxybis octane was not detected in any of the nipple-areola region samples and in only 20% of the para-axillary samples. Only isopropyl myristate was present at a high frequency in the samples after childbirth.

Relative Quantitative Analyses. The amounts of the five compounds relative to the most abundant compound in each sample also varied with sampling period in the two different body areas. The para-axillary area (Fig. 3) was characterized by a pattern with generally high levels of 1-dodecanol, 1-1′-oxybis octane, isocurcumenol, and α-hexyl-cinnamic aldehyde during pregnancy and a subsequent decline in abundance to nearly complete disappearance of these compounds after childbirth. The relative amount of isopropyl myristate in the para-axillary samples was high during pregnancy and increased after childbirth.

The nipple-areola area (Fig. 4) was characterized by a similar pattern but with greater relative amounts of isocurcumenol during late gestation and more variability in the relative amounts of isopropyl myristate during pregnancy.

Discussion

Our data showed that during pregnancy all women developed a distinctive olfactory pattern that involved at least five volatile compounds. This pattern was absent in samples from non-pregnant and non-lactating control subjects, but was present in samples taken during pregnancy as well as after childbirth. The compounds were present in samples from both the para-axillary and the nipple-areola regions. The precise time periods selected for sample collection were chosen on the basis of pragmatic criteria. The collection times that produced the above mentioned results were not fixed points but a series of 'windows' suitable for collection.

From a qualitative point of view, we can infer that α-hexyl-cinnamic aldehyde and isopropyl myristate remain constant like a matrix in the pattern; isocurcumenol functions as a distinctive signal between para-axillary and nipple-areola regions; and 1-1´-oxybis octane and 1-dodecanol behave as indicators for different periods of the pregnancy. Surprisingly, after childbirth, this pattern did not appear to be influenced by factors such as lactation or the menstrual cycle, but was modified by food and emotional anomalies. In particular, 1-1´-oxybis octane was affected by food anomalies; 1-1´-oxybis octane and 1-dodecanol were affected by emotional anomalies (data not shown).

From the relative quantitative results, we infer that isopropyl myristate represents a matrix of the pattern in both body regions and during all three sample periods, whereas 1-dodecanol, 1-1´-oxybis octane, isocurcumenol, and α-hexyl-cinnamic aldehyde behave as indicators for different phases of pregnancy; contemporaneously working as distinctive signals between the para-axillary and nipple-areola regions.

The presence of these volatile compounds and the related pheromonal role for some of them in other species may have implications from the point of view of evolution and phylogeny. First, three of the compounds identified in this study (oxybis octane, isocurcumenol, and α-hexyl-

cinnamic aldehyde) have not been found previously in any other animal species. Oxybis octane for instance has apparently never been found to have a significant biological role, and isocurcumenol is rather widespread but as a volatile compound produced by some plants (Ha et al., 2002; Yang et al., 2005; Zhou et al., 2007). α-Hexyl-cinnamic aldehyde has frequently been utilized as a fragrance in both cosmetic and detergent products (Rastogi et al., 1996; Kanei et al., 1999). Dodecanol and isopropyl myristate have been suggested previously to have a pheromonal role in both humans and other animal species. For example, 1-dodecanol has been identified as a volatile compound from the plant *Houttuynia cordata* Thunb. (Liang et al., 2005); as precursor of the main sex pheromone of the female of the crab, *Erimacrus isenbeckii* (Masuda et al., 2002); as a pheromone in the ano-genital marking of males and females of *Lemur catta* (Hayes et al., 2004); and as a volatile compound of human sweat (Meijerink et al., 2000, 2001). Isopropyl myristate is one of the main volatile compounds produced by the human axilla, and it is also often utilized as a component of deodorants and other cosmetic products (Labows et al., 1979). This compound is a volatile component of the head, thorax, and alimentary canal of the ant *Iridomyrmex humilis* (Cavill and Houghton, 1974), and as a sex pheromone that (together with heneicosane, docosane, nonacosane, and octadecanes) stimulates oviposition in the mosquito, *Aedes aegypti* (Corkum and Belanger, 2007).

On the basis of our results, we hypothesize that the distinctive chemical pattern of the para-axillary area could be useful for newborns to recognize their own mothers and distinguish them from other individuals. The pattern of volatiles from the nipple-areola region may function as an aid to finding sustenance. The results also demonstrate the effectiveness of the methodology. By using this collection and analysis method , we investigated the volatile compounds in a systematic way. Previously, such phenomena often have been investigated with inadequate methodologies, and as a consequence, the role of volatile compounds likely has been underestimated. The behavioral mechanisms related to these potential chemical signals bear future investigation in humans.

There are practical implications of the present research. The study of the mechanism of mother-child identification is important for the acquisition of new knowledge concerning the emission of signal molecules essential for mother-child identification, and also for setting the proper conditions for establishing solid mother-child bonding. An understanding of the mechanisms of newborn recognition of mothers also could have practical health implications. Moreover, the research provides a basis for testing hypotheses about the role of pheromones in the evolution and phylogeny of humans. It seems apparent that pheromones influence a wide range of reproductive behavior, a central theme to the survival of any species, a subject long underestimated for its impact on human behavior and evolution.

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Figure Legends

Fig. 1 TIC profile of a nipple-areola sample during pregnancy (red/bottom) compared to a similar sample from a non-pregnant and non-lactating control (black /top). The range corresponds to the elution of the five common compounds (1-dodecanol, R_t 17.83 min, **A**; 1-1′-oxybis octane, R_t T 21.60 min, **B**; isocurcumenol, R_t 21.79 min, **C**; α-hexyl-cinnamic aldehyde, R_t 23.27 min, **D**; isopropyl myristate, R_t 24.54 min, **E** . It focuses on the period between RT 17.60 min - 25.00 min that has been normalized. The unlabelled peaks reflect compounds not common to all samples.

Fig. 2 Comparison of relative frequency of occurrence of volatile compounds in samples from the para-axillary and nipple-areola regions from women (*N*=20) during three sampling periods.

Fig. 3 Relative amounts (mean ± 2 s.e., *N*=20) of five volatile compounds in samples from the para-axillary region during three sampling periods.

Fig. 4 Relative amounts (mean ± 2 s.e., *N*=20) of five volatile compounds in samples from the nipple-areola region during three sampling periods.