R.W. Dal Negro[1], M. Visconti[1], C. Micheletto[1], S. Tognella[1], M. Guerriero[2]

Reference urinary LTE_4 levels in normal individuals: a pilot study

[1] Lung Dept., Orlandi Gen. Hospital., Bussolengo - Verona, Italy - E-mail: rdalnegro@ulss22.ven.it
[2] ESI Dept., Statistical Section, University of Verona, Italy

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Corresponding author
Roberto W. Dal Negro
Lung Dept., Orlandi Gen. Hospital
Via Ospedale, 2
I-37012 Bussolengo, Verona, Italy
Tel: +39 045 6712193
Fax: +39 045 67122544
E-mail: rdalnegro@ulss22.ven.it

Summary

The definition of reference normal values for urinary LTE4 still represents an open question. ***Aim:*** *to assess the influence of gender and age on urinary LTE4 levels in normal individuals.* ***Methods:*** *after their informed consent, urinary LTE4 was measured in 124 well matched, non smoker, non atopic subjects (mean age 49.5 y±20.1 sd; range 4-85 y, 57 m;) without any clinically evident disease and not taking any drug for several months. In all subjects, urine were collected in the morning, and processed by an immunoenzimatic method (Cayman Chem, Mi, USA) via the Triturus system (Grifols, Spain).* ***Statistics:*** *t test, anova, linear regression, assuming p<0.05.* ***Results:*** *mean urinary LTE4 were 57.3 pg/ml in males (mean age 51.2 y±21.3 sd) and 57.0pg/ml in females (mean age 48.1 y±19.1 sd), p=ns. Linear regression showed no relationship between urinary LTE4 levels and subjects' age in the whole sample of subjects. When subjects were divided according to 4 different classes of age (0-14; 15-40; 41-60; >60), anova proved that mean urinary LTE4 levels were significantly different in the different classes of age, being higher in younger subjects (67.1 pg/ml ±33.4 sd; 69.8 pg/ml ±27.5 sd; 57.1 pg/ml ±25.4sd, and 45.1 pg/ml ±24.9, respectively) (anova p<.002; Welch test p<.005).* ***Conclusions:*** *1) gender does not affect urinary LTE4 levels in normals; 2) mean urinary LTE4 concentrations tend to a slight, but significant, decrease with the increase of the subjects' age, and this is clear in those over-60; 3) reference values for younger and older normal subjects (such as, under- and over-60 years) should be assumed accordingly.*

Introduction

Leukotrienes (LTs) have an established role in a wide variety of inflammatory diseases, including asthma, allergic rhinitis, atherosclerotic cardiovascular disease, inflammatory bowel disease, multiple sclerosis and cancer (1). LTs are metabolites of arachidonic acid and their synthesis can be triggered by a variety of soluble and particulate stimuli, including antigens, microbes, cytokines, immune complexes and model agonists such as calcium ionophores.

Leukotriene C4 (LTC4), the intracellular parent of the cysteinyl leukotrienes (cysLTs), is formed by conjugation of LTA4 and reduced glutathione by LTC4 synthase in mast cell, eosinophils, basophils, and macrophages (2). Of the 3 cysLTs, only LTE4 is stable enough to be detectable in extracellular fluids (3).

Urine is a biological fluid that is easy to collect with no significant risk of artifactual formation of LTs. The non-invasive sampling procedure also makes repeated collection possible from patients with different respiratory diseases, and the pattern of metabolites in urine probably reflects their whole body production (4). As the pulmonary metabolism of LTC4 results in a rapid formation of LTE4 with no further conversion, LTE4 can be regarded as the end product of cys-LTs in the lung and can work as appropiate marker or the systemic production of cys-LTs (5).
Independently of the particular method used for measuring eicosanoids (6-8), urine has been found the most suitable and convenient biological substrate for measuring the whole body production of cys-LTs in vivo (9-11). Nevertheless, only a few studies have focused the renal LTE4 excretion in healthy individuals, and the variability determinants (such as, gender, age), the knowledge in this area still remaining quite limited.
The aim of the present study was to assess the reference values for urinary LTE4 measured by a recently introduced EIA method as an alternative to radioimmunoassay and using acetylcholinesterase as label (12), in normal individuals ranging from childhood to elderly.

Subjects

Urinary LTE4 was measured in 124 normal subjects (57 males) from all over Italy after their informed consent: in particular, n=34 (27.4%) were from nord-western; n=31 (25.0%) from nord-eastern; n=29 (23.4%) from central regions, and n= 30 (24.2%) from southern regions. When subjects were too young to express their will officially (<18 years), the consent was required to their parents.
Subjects were invited by advertising, by random telephon calls, and by personal invitation. Exclusion criteria were: atopy and related syndromes (such as rhinitis, oculo-rhinitis, eczema and other skin troubles, intestinal disorders); atopic and non-atopic bronchial asthma; chronic obstructive pulmonary diseases; bronchiectasis; pneumonia; lung fibrosis and other granulomatous diseases; arthritis of any cause; cardiovascular, renal, neurological and gastroenteric diseases; malignancy.
Furthermore, subjects reporting any transitional, or acute inflammatory condition, or infection in the last 12 weeks were also excluded together to pregnant or lactating females, and subjects exposed to particular occupational risks known as able to induce long lasting local or systemic inflammatory conditions (such as exposition to TDI or phormaldeid).
Finally, smokers and subjects reporting any regular therapeutic treatment due to any cause were also excluded from the study.

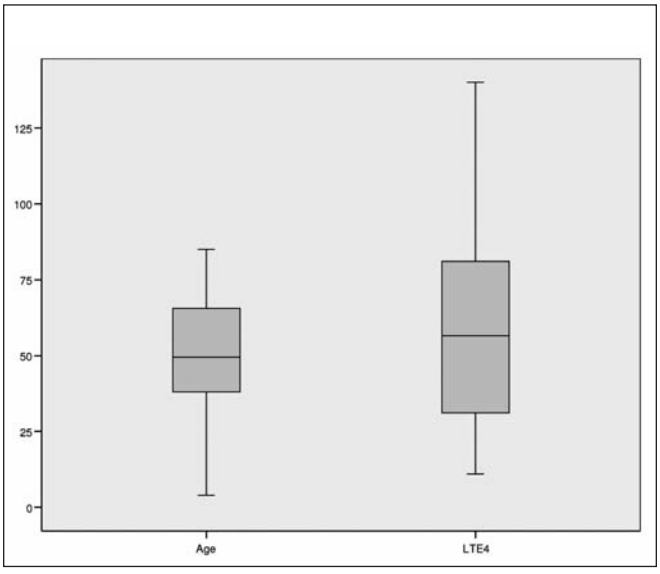
Methods

After their informed consent, urinary LTE4 was measured in 124 well matched, never-smoker, non atopic subjects (mean age 49.5y±20.1sd; range 4-85y, 57m;) without any clinically known and apparent relevant disease, and not taking any drug over at least 6 months. In all subjects, urine were collected in the morning (at 8 am), and processed by enzyme immunoassay (ACETM Competitive Enzyme Immunoassay, Cayman Chemical, Ann Arbor, Mich, USA), as reported by Pradelles et al (12), via the Triturus System (Grifols, Spain).
This assay is based on the competition between LTE4 and an LTE4- acetylcholinesterase (AChE) conjugate (LTE4 tracer) for a limited amount of LTE4 antiserum. Because the concentration of the LTE4 tracer is held constant while the concentration of LTE4 varies, the amount of LTE4 tracer that is able to blind to the LTE4 antiserum will be inversely proportional to the concentration of LTE4 in the well. This antibody-LTE4 complex blinds to a mouse monoclonal anti-rabbit IgG that has been previously attached to the well. The plate is washed to remove any unbound reagents and then Ellman's reagent (which contains the substrate to AChE) is added to the well. This reagent consists of acetylthiocholine and 5,5'-dithio-bis-(2-nitrobenzoic acid). Hydrolysis of acetylthiocholine by AChE produces thiocholine. The non-enzymatc reaction of thiocholine with 5,5'-dithio-bis-(2-nitrobenzoic acid) produces 5-thio-2-nitrobenzoic acid, which has a strong absorbance at 412 nm. AChE has several advantages over other enzymes commonly used for enzyme immunoassays. Unlike horseradish peroxidase, AChE does not self-inactivate during turnover. In addition, the enzyme is highly stable under the essay conditions, has a wide pH range (pH 5-10), and is not inhibited by common buffer salts and preservatives. The product of this enzymatic reaction has a distinct yellow colour and absorbs strongly at 412 nm. The intensity of this colour, determined spectrophotometrically, is proportional to the amount of LTE4 tracer bound to the well, which is inversely proportional to the amount of free LTE4 present in the well. LTE4 was measured by enzyme immunoassay on all samples according to the manufacturers' instructions and expressed in pg/mg creatinine (pg/mg).

Table 1 - Comparison of both age and urine LTE4 values by gender

	gender	n	mean	sd	p-value
LTE_4	male	57	57,25	29,551	0,97
	female	67	57,05	26,574	
Age	male	57	51,19	21,347	0,40
	female	67	48,07	19,112	

Figure 1 - Boxplot for age and urine LTE4 values



Statistics

As the distribution of urinary LTE4 values in the population sample was normal (Kolmogorov-Smirnov test), the inferential analysis (t test) was used to check if the mean urinary LTE4 values were significantly affected by the subjects' gender (such as males vs females).
Subjects were also divided into four classes of age (A: 0-14 y; B: 15-40y; C: 41-60y, and D: >60y of age) in order to investigate any possible relationship with the subjects' age.
Regression analysis was used to assess the consistency of linear relationships between the age and the urinary LTE4 values, by stratifying for the subjects' gender.
Analysis of variance and Games-Howell procedure for multiple comparisons were used to investigate the changes of LTE4 values within the four different classes of age and the possible existence of significant relationships with any class of those.
The significance level assumed was $p<0.05$.

Results

The overall sample of subjects consisted in 124 subjects, 57 males and 67 females (46% and 54%, respectively).
The whole sample had a mean age of 49.5y ± 20.1 sd, (range 4-85 y, median 49.5 y), while the corresponding mean urinary LTE4 value was 57.1 pg/ml ± 27.9 sd (range 11-140 pg/ml, median 56.5 pg/ml); mean LTE4 values were not different when subject's geographic origin was considered (p=ns). The dispersion of urinary LTE4 values around its mean value was not negligible, even though no real outliers were observed (Figure 1).
No significant difference was assessed for both age and urinary LTE4 values by gender in the whole sample, and the corresponding differences between means were 0.197 pg/ml (p=0.97) and 3.118 y, (p=0.40), respectively (Table 1). In particular, the regression analysis proved a slight negative relationship (p=0,001; r = -0,33). between age and urinary LTE4 in the whole sample of subjects.
Acceptable results were achieved in terms of relative consistency of each class when the whole sample was clustered into four classes of age, such as: 0-14 y = 9.7%; 15-40 y = 21.8; 41-60 = 37.9%, and >60 = 30.6% subjects, respectively.
When the analysis of variance was calculated within the four different classes of age it was proved that the corresponding mean LTE4 values were significantly different (Table 2A; 2B, and Figure 2). Moreover, when the Games-Howell procedure for multiple comparisons was used, the pairs of measures significantly different were those corresponding to the groups 15-40 years and >60 years.
Furthermore, the t-test which compared mean values for urinary LTE4 and age by gender lead to the conclusion that those variables were not affected by the subjects' gender (Table 3).
The linear regression calculated within the different classes of age (urinary LTE4 as the dependent variable, and age as the independent variable) proved that a more strict relationship between these variables was existing for younger subjects only (Table 4A and 4B). When the regression was

Table 2A - Subjects' n.; mean urine LTE4 values, sd; es; CI 95%; min. and max. values in the four different classes of age and in the total sample of subjects

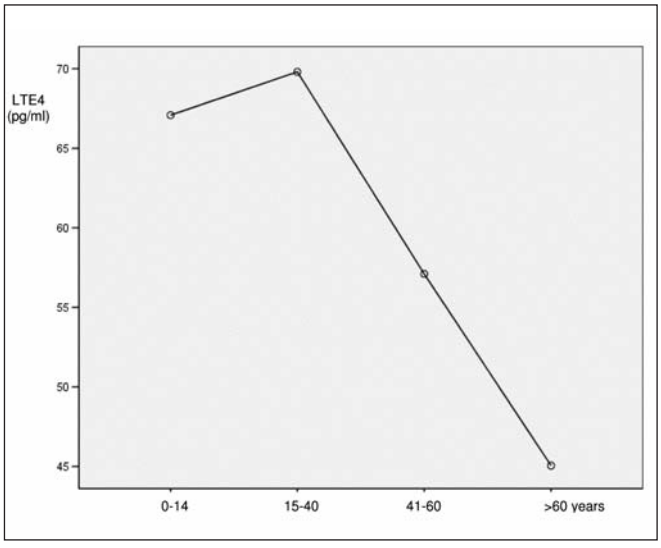
	n	mean	sd	CI 95%		min.	max
				Lower Limit	Upper Limit		
0-14 y (A)	12	67,08	33,438	45,84	88,33	22	140
15-40 y (B)	27	69,80	27,462	58,94	80,67	15	119
41-60 y (C)	47	57,10	25,356	49,66	64,54	17	110
> 60 y (D)	38	45,06	24,913	36,87	53,24	11	96
Total	124	57,14	27,866	52,19	62,09	11	140

Table 2B - Urine LTE4 concentrations: multiple comparison of means by age

means	p *
A,B (67.08 vs 69.80)	> 0 . 0 5
A,C (67.08 vs 57.10)	> 0 . 0 5
A,D (67.08 vs 45.06)	> 0 . 0 5
B,C (69.80 vs 57.10)	> 0 . 0 5
B,D (69.80 vs 45.06)	< 0 . 0 1
C,D (57.10 vs 45.06)	> 0 . 0 5

** (Games-Howell procedure).*

Figure 2 - Mean urine LTE4 concentrations in the different classes of age



calculated only within the pediatric age (0-14 years), the value of the linear relationship changed from -0.33 (that of the whole sample) to -0.54, with a p value close to the statistical significance (p=0.068).

From a general point of view, the subjects belonging to the 15-40 y and the >60y class of age seem characterized by substantially different urinary LTE4 mean values, actually their corresponding values for CI 95% proving not overlapping (Table 5).

Discussion

Once released, the metabolic cascade of arachidonic acid gives rise to a group of compounds known as eicosanoids, and leukotrienes (LTs) represent a substantial proportion of these compounds.

In human eosinophils, mast cells, and basophils, LTA4 is conjugated with reduced glutathione by LTC4-synthase to produce LTC4, the first of the cys-LTs. Of the cys-LTs, only LTE4, which is an extracellular metabolite, has been proved to be stable enough to be detectable in the extracellular fluids, and particularly in urine.

Cys-LTs expression is increased in several pathological conditions, and particularly in bronchial asthma and some related conditions, where the amount of LTE4 is five- to ten-fold greater than in normal individuals (13-20), likely due to the upregulation of 5-LO and FLAP messenger RNA in asthmatic subjects (14). Actually, in a previous study carried out on a series of 386 asthma patients, mean urinary LTE4 levels proved significantly higher than those measured in normals assessed in the present study, and increasing according to their asthma severity (such as: 129.1 pg/ml ± 74.8 sd in mild; 330.7 pg/ml ± 72.3 sd in moderate, and 432.3 pg/ml ± 88.1 sd in severe asthma) (21). In a

Table 3 - Comparisons (t test) between urine LTE4 concentrations in the different classes of age by gender and age

Class of age		gender	n	mean	ds
0-14 y	LTE4	male	6	69,50	38,713
		female	6	64,67	30,774
	Age	male	6	10,17	3,312
		female	6	11,33	2,503
15-40 y	LTE4	male	9	70,67	30,116
		female	18	69,37	26,944
	Age	male	9	31,00	8,660
		female	18	33,50	6,501
41-60 y	LTE4	male	21	63,86	26,529
		female	26	51,64	23,466
	Age	male	21	50,29	5,943
		female	26	50,50	4,420
>60 y	LTE4	male	21	41,39	23,829
		female	17	49,59	26,192
	Age	male	21	72,48	6,555
		female	17	72,76	5,178

Table 4 A - Linear regression within the different classes of age with LTE4 as the dependent variable and age as the independent variable

Class of age	Model	Sum of squares	df	Mean of squares	F	Sig.
0-14 anni 1	Regression	3634,821	1	3634,821	4,195	,068(a)
	Residual	8664,096	10	866,410		
	Total	12298,917	11			
15-40 y 1	Regression	352,702	1	352,702	,458	,505(a)
	Residual	19254,847	25	770,194		
	Total	19607,550	26			
41-60 y 1	Regression	125,491	1	125,491	,192	,664(a)
	Residual	29449,989	45	654,444		
	Total	29575,480	46			
> 60 y 1	Regression	22,385	1	22,385	,035	,852(a)
	Residual	22942,509	36	637,292		
	Total	22964,894	37			

further study designed for investigating the difference in nasal response between ASA-tolerant and ASA-intolerant asthmatics, both subsets of subjects confirmed their high basal mean urinary LTE4 concentrations when compared to normals (such as: 333.1 pg/ml ± 202.8 sd and 433.0 pg/ml ± 361.7 sd, respectively (20).

In general terms, even if the basal production of cys-LTs contributes to depict the asthma phenotype, genetic variability in 5-LO biosynthetic and receptor pathway gene loci may affect cys-LTs production in humans (15). Actually, some biosynthetic polymorphisms (such as, cysLTR2 and ALOX5) may predispose particular clusters of individ-

Table 4B –

Class of age	Model		Not standardized coefficients B	es	Standardized coefficienti Beta	t	Sig.
0-14 y	1	(Constant)	135,306	34,375		3,936	,003
		Age	-6,346	3,098	-,544	-2,048	,068
15-40 y	1	(Constant)	86,464	25,192		3,432	,002
		Age	-,510	,754	-,134	-,677	,505
41-60 y	1	(Constant)	73,432	37,482		1,959	,056
		Age	-,324	,740	-,065	-,438	,664
>60 y	1	(Constant)	54,623	51,214		1,067	,293
		Age	-,132	,703	-,031	-,187	,852

Dependent variable: LTE4

uals to excessive cys-LTs concentrations who can represent a further phenotype (22). The presence of these biosynthetic polymorphisms may likely explain the variability of urinary LTE4 concentrations also in healthy humans as assessed in real life in the present study.

Further to the genetic sources of variability, also host (i.e.: age, gender) and environmental (i.e.: smoking habit) factors can be presumed to influence LTs, and particularly LTE4 concentrations in urine, the intrinsic complexity of biological measures to perform representing a further source of variability, particularly in measuring LTE4 levels in urine.

The immunoassay based on radioactivity (RIA) or on enzyme activity (EIA) is regarded as a rapid, sensitive and suitable method for measuring different eicosanoid concentrations (6). EIA or RIA analyses have been most commonly performed in combination with reverse phase-high performance liquid chromatography (RP-HPLC) (7), even though measurements of urinary LTE4 by means of RP-HPLC alone are not regarded as an alternative due to the low sensitivity of the detection via the ultra-violet absorbance. Moreover, the gas chromatography-mass spectrometry (GC-MS) is a method highly sensitive and specific which could theoretically represent an alternative for the measurement of urinary LTE4, but all mass spectrometric procedures demand cumbersome and time-consuming purification of biological samples (8).

In the present study, a recently introduced EIA method (12) was employed as an alternative to radioimmunoassay, in order to lower both the technical complexity and costs.

Reference for urine LTE4 concentrations in normal humans from childhood to elderly were still missing to our knowledge, and the present study was specifically oriented to assess the role of age and gender in affecting urine LTE4 variability.

Table 5 – Range of urinary LTE4 mean reference values in the whole sample of normal subjects, and in the four different classes of age (CI -95%)

LTE4	Mean (pg/ml)	Lower limit (pg/ml)	upper limit (pg/ml)
Whole sample	57.1	52.2	62.1
0 -14 y	67.1	45.8	88.3
15 - 40 y	69.8	58.9	80.7
41 - 60 y	57.1	49.7	64.5
>60 y	45.1	36.9	53.2

Firstly, LTE4 measurements were absolutely independent of the patients' gender, and then the same reference values would be assumed for normal males and females.

On the contrary, urine TLE4 concentrations proved to decrease progressively, even though slightly, in proportion to the subjects' age, being this trend more pronounced when subjects were clustered in four different classes of age. Actually, as those under- and those over-60 years showed both mean values and CI 95% for LTE4 concentrations different enough to be discriminant, the need of different normal reference values based on the subjects' age is thus suggested.

Finally, the strict assessment of the main confounding factors (such as gender and age) presumed as able to affect the extent of LTE4 variability in normal individuals is considered a crucial step in order to investigate and define more precisely the changes in urine LTE4 concentrations in several pathological conditions and/or following specific anti-LTs treatments.

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