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RESEARCH ARTICLE

Urinary biomarkers suggest that estrogen-DNA adducts may play a role in the aetiology of non-Hodgkin lymphoma

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Abstract

A variety of evidence suggests that estrogens may induce non-Hodgkin lymphoma (NHL). The reaction of catechol estrogen quinones with DNA to form depurinating estrogen-DNA adducts is hypothesized to initiate this process. These adducts are released from DNA, shed from cells into the bloodstream and excreted in urine. The aim of this study was to determine whether or not the depurinating estrogen-DNA adducts might be involved in the aetiology of human NHL. Estrogen metabolites, conjugates and depurinating DNA adducts were identified and quantified in spot urine samples from 15 men with NHL and 30 healthy control men by using ultraperformance liquid chromatography/tandem mass spectrometry. The levels of estrogen-DNA adducts were significantly higher in the men with NHL than in the healthy control men. Thus, formation of estrogen-DNA adducts may play a critical role in the aetiology of NHL, and these adducts could be potential biomarkers of NHL risk.

Keywords: *Non-Hodgkin lymphoma; depurinating estrogen-DNA adducts; urinary biomarkers; solid-phase extraction*

Introduction

In recent decades, there has been an increase worldwide in the incidence of non-Hodgkin lymphoma (NHL) among both men and women (Muller et al. 2005, Grulich & Vajdic 2005, Ekstrom-Smedby 2006, Alexander et al. 2007). The American Cancer Society estimates that, in 2009, 65 980 new cases of NHL will be diagnosed in the United States, representing the fifth most common malignancy, and 19 500 people are expected to die of this disease (American Cancer Society 2009; http://www.cancer.org). NHL consists of more than 40 different lymphoproliferative malignancies which arise from B cells, T cells or natural killer (NK) cells (Swerdlow et al. 2008). The risk factors for developing NHL include immunosuppression, various infectious and autoimmune disorders, a family history of haematopoietic malignancy, and certain occupational exposures, including pesticides and solvents (Muller et al. 2005, Grulich & Vajdic 2005, Ekstrom-Smedby 2006, Alexander et al. 2007).

At first, exposure to benzene was associated only with an increased risk of acute myeloid leukaemia (Hayes et al. 1997), but the induction of NHL by benzene has now also become apparent (O'Connor et al. 1999, Mehlman 2006, Miligi et al. 2006, Smith et al. 2007, Zhang et al. 2007, Steinmaus et al. 2008). Benzene induces NHL in experimental studies of mice and rats (Farris et al. 1993, Huff et al. 1989). Occupational studies of solvent exposures suggest that initiation of NHL is the result of DNA

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damage and subsequent defects in DNA repair systems (Zhang et al. 2007, Tompa et al. 1994, Biro et al. 2002).

Estrogen compounds contain a benzene ring and we have previously shown that estrogens can be bioactivated to catechol quinones, analogous to the bioactivation of benzene (Cavalieri et al. 2002b). The catechol quinones of both benzene and estradiol (E_2) induce the proliferation of human blood mononuclear cells, including those that give rise to myeloid leukaemia and NHL (Chakravarti et al. 2006). The catechol quinone of benzene reacts with DNA to form depurinating N3Ade and N7Gua adducts (Figure 1) (Cavalieri et al. 2002b), analogous to the adducts that are formed by the catechol quinones of estrone (E_1) and E_2 (Figure 2) (Cavalieri et al. 1997, Li et al. 2004, Zahid et al. 2006). In fact, treatment of human blood mononuclear cells with the *ortho*-quinone of benzene or E_2 led to the formation of the corresponding N3Ade and N7Gua adducts (Chakravarti et al. 2006).

Animal studies in rats and mice have shown that estrogens affect lymphoid tissues (Li 1996, Forsberg 1984). Polymorphisms in CYP17 and CYP19, which are involved in the biosynthesis of estrogens, and catechol-*O*-methyltransferase, which methylates catechol estrogens, have been associated with increased risk of NHL in humans (Skibola et al. 2005a, b, 2007, 2008). Interestingly, some studies have also suggested that estrogen replacement therapy may be a risk factor for NHL in women (Bernstein & Ross 1992, Cerhan et al. 2002).

Our laboratory has extensively investigated the molecular mechanisms of estrogen carcinogenesis and provided ample evidence for the formation of depurinating estrogen-DNA adducts, which are formed via reaction of estrogen quinones with DNA. The predominant depurinating estrogen-DNA adducts, 4-$OHE_1(E_2)$-1-N3Ade and 4-$OHE_1(E_2)$-1-N7Gua (Cavalieri et al. 1997, Li et al. 2004), are shed from cells into the bloodstream and, eventually, are excreted in urine, where they can be analysed, along with estrogen metabolites and conjugates (Gaikwad et al. 2008, 2009a). Release of the depurinating adducts leaves apurinic sites in the DNA, which, in turn, induce mutations. It is thought that critical mutations generated by specific DNA damage can result in abnormal cell proliferation leading to cancer (Chakravarti et al 2001, Cavalieri et al. 2006, Fernandez et al. 2006, Mailander et al. 2006, Zhao et al. 2006, Gaikwad et al. 2008, 2009a). All of these results support the hypothesis that estrogens could initiate NHL through the formation of depurinating DNA adducts.

Considering the resemblance of bioactivation of endogenous estrogens and benzene, their effects on lymphoid tissue in animals (Li 1996, Forsberg 1984), association of polymorphisms in estrogen biosynthesis and metabolism with NHL in humans (Skibola et al. 2005a, b, 2007, 2008), our recent success in establishing the chain of reactions that lead to estrogen carcinogenesis (Gaikwad et al. 2008, 2009a) and the discovery that the level of estrogen-DNA adducts in urine correlates with risk of developing breast cancer (Gaikwad et al. 2008, 2009a), we were interested to test the hypothesis that depurinating DNA adducts may be involved in the aetiology of NHL. The results presented in this article suggest that the levels of estrogen metabolites, conjugates and depurinating DNA adducts differ significantly between healthy men and men with NHL. The ratios of depurinating DNA adducts to their respective estrogen metabolites and conjugates were significantly associated

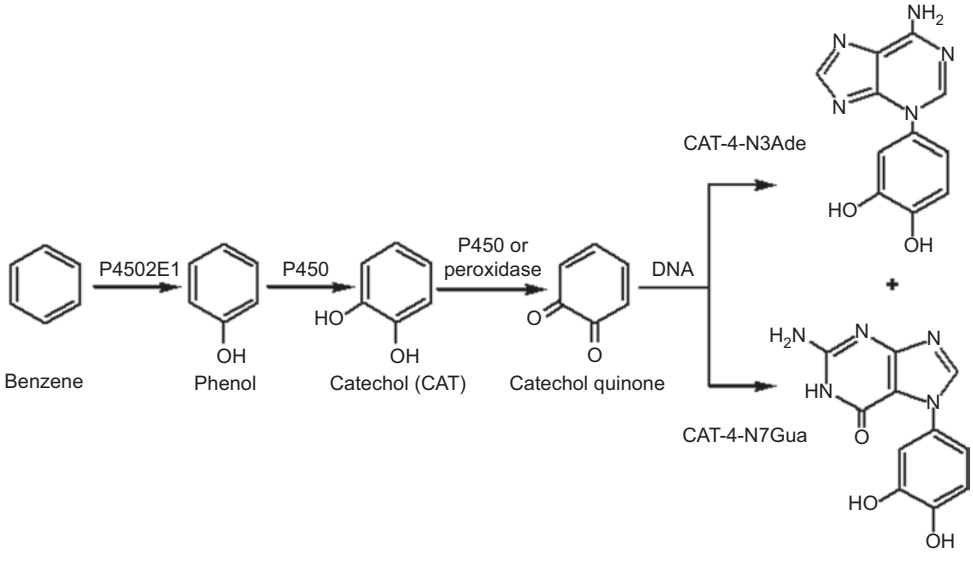

Figure 1. Oxidative metabolism of benzene to form depurinating DNA adducts. Metabolic conversion of benzene yields the phenol and then the catechol (CAT), which is further oxidized to catechol quinone. This quinone reacts with DNA to form the two depurinating DNA adducts.

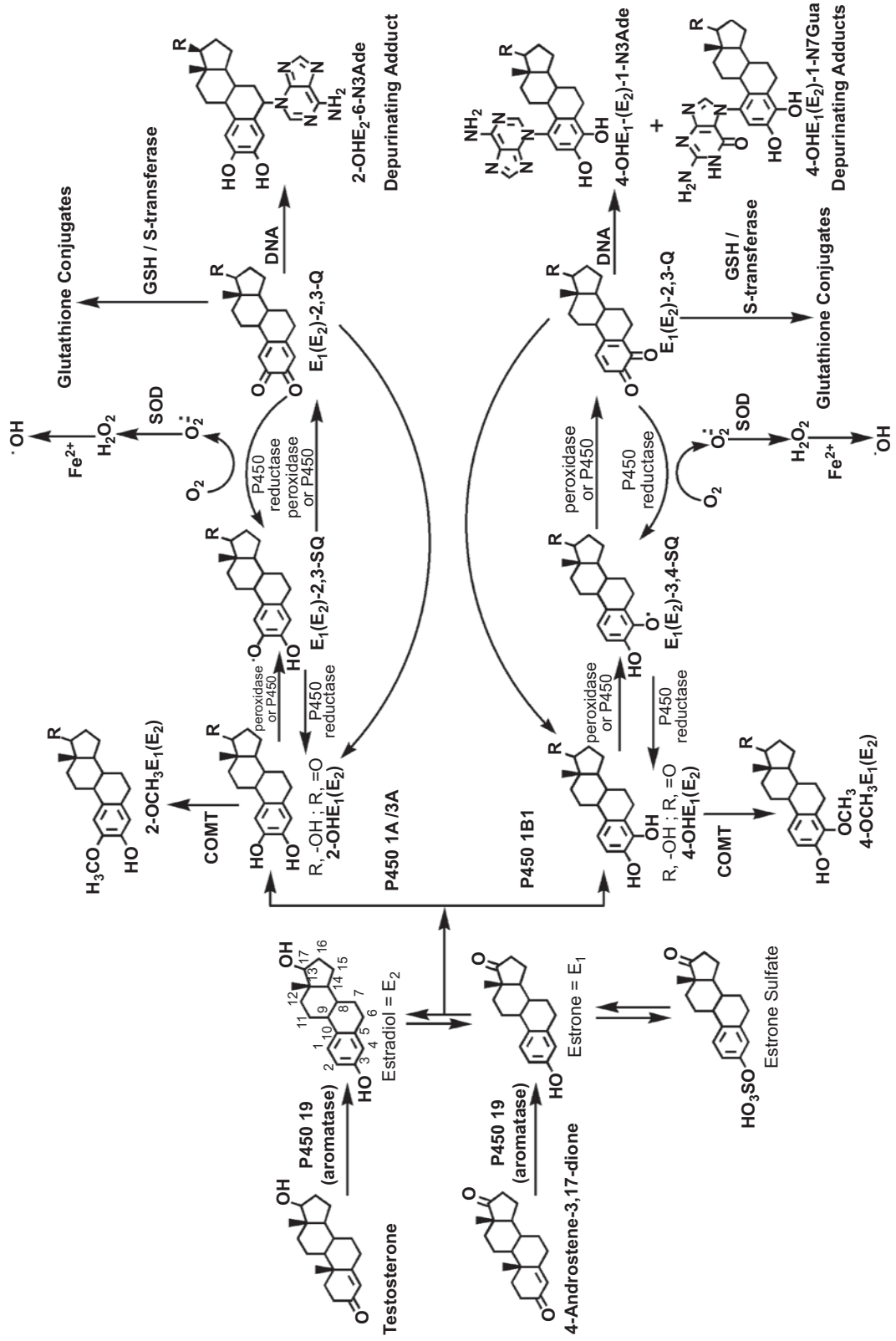

Figure 2. Biosynthesis and metabolic activation of the estrogens E_1 and E_2. Metabolic activation of E_1 and E_2 leads to 2- and 4-catechol derivatives, which further oxidize to yield the corresponding reactive quinones. The quinones react with DNA to form depurinating DNA adducts. In the deactivation pathway, which operates in parallel, the catechol derivatives are methylated to form methoxy catechol estrogens; in addition, the quinones are reduced by quinone reductase, as well as conjugated with GSH, and, thus, are rendered harmless. The shift in the apparent balance between these activating and deactivating pathways towards formation of depurinating DNA adducts could lead to the initiation of non-Hodgkin lymphoma.

with health status. We further suggest that depurinating estrogen-DNA adducts should be evaluated as potential biomarkers for NHL risk in future studies.

Materials and methods

Materials

Phenyl solid-phase extraction (SPE) cartridges were purchased from Varian (Palo Alto, CA, USA). Androstenedione 1, testosterone 2, E_1-sulfate 3, E_2 4, E_1 5, 2-hydroxyestradiol (2-OHE_2) 6, 2-OHE_1 7, 16α-OHE_2 10, 16α-OHE_1 11, 2-methoxyestradiol (2-OCH_3E_2) 12, 2-OCH_3E_1 13, 4-OCH_3E_2 14, 4-OCH_3E_1 15, 2-OH-3-OCH_3E_2 16 and 2-OH-3-OCH_3E_1 17 were purchased from Steraloids Inc. (Newport, RI, USA). 4-OHE_2 8 and 4-OHE_1 9 were synthesized as previously described (Saeed et al. 2005). 2-OHE_2-1-glutathione (SG) 18, 2-OHE_2-4-SG 19, 2-OHE_1-1-SG 20, 2-OHE_1-4-SG 21, 2-OHE_2-(1+4)-cysteine (Cys) 22, 2-OHE_1-1-Cys 23, 2-OHE_1-4-Cys 24, 2-OHE_2-1-*N*-acetylcysteine (NAcCys) 25, 2-OHE_2-4-NAcCys 26, 2-OHE_1-1-NAcCys 27, 2-OHE_1-4-NAcCys 28, 4-OHE_2-2-SG 29, 4-OHE_1-2-SG 30, 4-OHE_2-2-Cys 31, 4-OHE_1-2-Cys 32, 4-OHE_2-2-NAcCys 33 and 4-OHE_1-2-NAcCys 34 were synthesized by using the procedure of Cao et al. (1998). 4-OHE_2-1-N7Gua 35, 4-OHE_1-1-N7Gua 36, 4-OHE_2-1-N3Ade 37, 4-OHE_1-1-N3Ade 38, 2-OHE_2-6-N3Ade 39 and 2-OHE_1-6-N3Ade 40 were synthesized by following the reported methods (Li et al. 2004, Stack et al. 1996, Zahid et al. 2006). All solvents were high-performance liquid chromatography (HPLC) grade and all other chemicals used were of the highest grade available.

Study population

We collected 45 urine samples from two sources. Fifteen men with NHL were recruited in the Lymphoma Clinic at the University of Nebraska Medical Center (UNMC) (Table 1). Men with NHL were recruited between August 2005 and November 2007, and their ages ranged between 49 and 88 years. For each NHL sample, two age-matched healthy men were used as controls. Thirty healthy men, ages between 50 and 78 years, were recruited at the University of Buffalo as part of the Western New York Health Cohort Study. The median ages of NHL cases and controls were 59 and 60 years, respectively. All procedures were approved by the UNMC Institutional Review Board. Signed consents included authorization to collect and bank urine samples and collect demographic and clinical information.

Sample collection

A spot urine sample of about 50 ml was collected from each participant and 1 mg ml^{-1} ascorbic acid was added

Table 1. Patient characteristics, treatment and follow-up.

NHLcase	Age (years)	NHL type	Prior chemotherapy	Follow-up
1	55	Diffuse large B-cell lymphoma	CHOP-R/XRT	Alive – complete remission
2	57	T-cell lymphoblastic lymphoma	LBL-1/HCVAD/ICE/Pento /TBI	Dead of the disease
3	61	B-cell small lymphocytic lymphoma	Fludara/bu/flu/Thymo	Alive – complete remission
4	59	Peripheral T-cell lymphoma	CHOP/HCVAD/ESHAP	Dead of the disease
5	62	Follicular B-cell lymphoma	Multiple/lenalidomide	Alive – no evidence of disease
6	64	Diffuse large B-cell lymphoma	CHOP-R	Alive – no evidence of disease
7	61	Follicular and diffuse large B-cell lymphoma	CHOP-R/PSCT/Bexxar	Alive – no evidence of disease
8	60	Marginal zone B-cell lymphoma	CHOP-R/XRT/CHOP-R	Alive – with relapse
9	63	Mantle B-cell lymphoma	CHOP-R Velcade	Alive – no evidence of disease
10	88	Diffuse large B-cell lymphoma	CHOP-R	Alive – no evidence of disease
11	61	T-cell prolymphocytic leukaemia	CHOP/HyperCVA	Dead of the disease
12	49	Cutaneous T-cell lymphoma	CHOP/ICE	Dead of the disease
13	55	Diffuse large B-cell lymphoma	CHOP-R	Alive – complete remission
14	56	Follicular B-cell lymphoma	CHOP-R/MaGrath/PSCT	Alive – relapse NHL
15	60	Follicular and diffuse large B-cell lymphoma	CHOP-R/Rice/PSCT/relase	Alive – with NHL

NHL, non-Hodgkin lymphoma; CHOP-R, cyclophosphamide, doxorubicin, oncovin, prednisone, rituximab; HCVAD, cyclophosphamide, doxorubicin, oncovin, prednisone, methotrexate, cytarabine; RICE, ifosfamide, carboplatin, etoposide, rituximab; PSCT, autologous stem cell transplant; XRT, involved field irradiation; MaGrath, cyclophosphamide, doxorubicin, oncovin, methotrexate, ifosfamide, etoposide, cytarabine; LBL-1, cyclophosphamide, doxorubicin, prednisone, vincristine, maintenance 6MP and L-asparaginase; Pento/TBI, miniallogeneic stem cell transplant with pentostatin/total body irradiation; fludara, fludarabine; bu/flu/Thymo, busulfan/fludarabine/thymoglobulin and allogeneic stem cell transplant; ESHAP, etoposide, cytarabine, cisplatin, SoluMedrol.

to prevent oxidation of the catechol moieties in the various estrogen compounds. The urine samples were aliquoted, frozen and four 10-ml aliquots were transferred to the Eppley Institute, UNMC, on dry ice and were stored at -80°C until analysis. Thus, each analytical sample was thawed only once prior to analysis.

Solid-phase extraction of urine

The SPE method development and validation were described previously (Gaikwad et al. 2008). Briefly, after adjusting 2-ml aliquots of urine samples to pH 7, they were loaded onto 100-mg phenyl cartridges preconditioned with methanol and the loading buffer, 10 mM ammonium formate, pH 7. The cartridges were washed with the loading buffer, and then the compounds of interest were eluted from the cartridge by using an elution buffer, methanol/10 mM ammonium formate, pH 7 (90:10) with 1% acetic acid. The eluates from both the experimental and control samples were concentrated and subjected to ultraperformance liquid chromatography/tandem mass spectrometry (UPLC/MS-MS) analysis.

We previously found that the treatment of urine with glucuronidase/sulfatase led to significant increases (10–20-fold) in the levels of E_1 and E_2; however, the levels of estrogen metabolites, conjugates and adducts changed marginally and in many cases decreased because of the incubation for 8 h at 37 °C (Gaikwad et al. 2008). Thus, to avoid artefacts and errors that are introduced by maintaining the urine samples at 37°C for 8 h, we carried out all the analyses without treating the samples with glucuronidase/sulfatase. For this reason, the reported levels of E_1 and E_2 are 10–15 times less than the total values.

UPLC/MS-MS analysis of urine samples

All analyses were performed on a Waters (Milford, MA, USA) Quattro Micro triple quadrupole mass spectrometer by using electrospray ionization (ESI) in positive ion (PI) and negative ion (NI) mode, with an ESI-MS capillary voltage of 3.0 kV, an extractor cone voltage of 2 V, and a detector voltage of 650 V. Desolvation and cone gas flow were maintained at 400 and 60 l h^{-1}, respectively. Desolvation temperature and source temperature were set to 200 and 100°C, respectively. For all the studies, a methanol:water (1:1) mixture with 0.1% formic acid was used as the carrier solution. The parent and daughter ion data obtained for all standard compounds were used to generate the multiple reaction monitoring (MRM) method for UPLC/MS-MS operation (Gaikwad et al. 2008).

UPLC/MS analyses of estrogen-related compounds (Table 2) in urine extracts were carried out with a Waters Acquity UPLC system connected with the

Table 2. Representative metabolic profile of a urine sample obtained from a man with non-Hodgkin lymphoma (NHL)[a].

No.	Compound	Total (pmol mg^{-1} creatinine)
1	Androstenedione	4.7
2	Testosterone	0.1
3	E_1-Sulfate	0.9
4	E_2[b]	24.8
5	E_1[b]	
6	2-OHE_2	2.2
7	2-OHE_1	
8	4-OHE_2	8.3
9	4-OHE_1	
10	16α-OHE_2	31.4
11	16α-OHE_1	
12	2-OCH_3E_2	22.1
13	2-OCH_3E_1	
14	4-OCH_3E_2	3.2
15	4-OCH_3E_1	
16	2-OH-3-OCH_3E_2	0
17	2-OH-3-OCH_3E_1	
18	2-OHE_2-1-SG	4.8
19	2-OHE_2-4-SG	
20	2-OHE_1-1-SG	
21	2-OHE_1-4-SG	
22	2-OHE_2-1+4-Cys	
23	2-OHE_1-1-Cys	
24	2-OHE_1-4-Cys	
25	2-OHE_2-1-NAcCys	
26	2-OHE_2-4-NAcCys	
27	2-OHE_1-1-NAcCys	
28	2-OHE_1-4-NAcCys	
29	4-OHE_2-2-SG	1.1
30	4-OHE_1-2-SG	
31	4-OHE_2-2-Cys	
32	4-OHE_1-2-Cys	
33	4-OHE_2-2-NAcCys	
34	4-OHE_1-2-NAcCys	
35	4-OHE_2-1-N7Gua	0.71
36	4-OHE_1-1-N7Gua	
37	4-OHE_2-1-N3Ade	0.95
38	4-OHE_1-1-N3Ade	
39	2-OHE_2-6-N3Ade	0.08
40	2-OHE_1-6-N3Ade	
(Ratio-4) + (Ratio-2) x 1000		131

[a]Typically, each 2-ml urine sample was analysed at least two times. The data obtained from LC/MS-MS were processed and normalized to creatinine levels. As the E_1 and E_2 derivatives are interconvertible, the total amount for each E_1 plus E_2 derivative in the various categories are presented in the last column and used for calculating the final ratios of depurinating adducts to the respective metabolites and conjugates (Gaikwad et al. 2008). 4-$OHE_1(E_2)$-1-N3Ade + 4-$OHE_1(E_2)$-1-N7Gua/4-catechol estrogens + 4-catechol estrogen conjugates = no. 37 + 38 + 35 + 36/no. 8 + 9+14 + 15 + 29 through 3 42-$OHE_1(E_2)$-6-N3Ade/2-catechol estrogens + 2-catechol estrogen conjugates = no. 39 + 40/no. 6 + 7+12 + 13 + 18 through 28.

[b]Free E_2 and E_1 in the urine sample.

high-performance Quattro Micro triple quadrupole mass spectrometer. Analytical separations on the UPLC system were conducted using an Acquity UPLC BEH C18 1.7 µm column (1 x 100 mm) at a flow rate of 0. 15 ml min^{-1}. The gradient started with 80% A (0.1% formic acid in H_2O) and 20% B (0.1% formic acid in CH_3CN), changed to 79% A over 4 min, followed by a 6-min linear gradient to 45% A, resulting in a total separation time of 10 min. The elutions from the UPLC column were introduced to the Quattro Micro mass spectrometer. Resulting data were processed by using QuanLynx software (Waters) to quantify the estrogen metabolites. The precision of these analyses is provided by the limit of detection and coefficient of variation for the UPLC-MS/MS method, which were presented earlier (Gaikwad et al. 2008).

A batch of 10 random samples was run in triplicate during each UPLC-MS/MS analysis. For each batch of samples, pure standards were used to optimize the

Table 3. Urinary levels of estrogen compounds in men with non-Hodgkin lymphoma (NHL) and health control men[a].

		Healthy controls (pmol mg^{-1} creatinine) (n = 30)			NHL (pmol mg^{-1} creatinine) (n = 15)			
No.	Compound	Median	Min	Max	Median	Min	Max	p-Value[b]
1	Androstenedione	7.9	1.95	21.59	1.4	0.00	6.76	<0.0001
2	Testosterone	**2.1**	0.13	9.21	**0.1**	0.00	1.40	<0.0001
3	E_2	**11.6**	0.00	33.63	**9.8**	0.40	34.25	
4	E_1							
5	E_1-Sulfate	**0.9**	0.00	11.69	**1.4**	0.13	6.50	
6	2-OHE_2	**7.8**	0.98	36.08	**6.6**	0.00	93.89	
7	2-OHE_1							
8	4-OHE_2	**7.3**	0.96	40.26	**8.2**	0.05	147.59	
9	4-OHE_1							
10	16α-OHE_2	**128.8**	9.89	402.44	**31.4**	6.80	706.53	
11	16α-OHE_1							
12	2-OCH_3E_2	**22.9**	0.62	290.47	**20.1**	1.06	163.44	
13	2-OCH_3E_1							
14	4-OCH_3E_2	**15.0**	2.13	274.97	**4.0**	0.00	147.86	0.0087
15	4-OCH_3E_1							
16	2-OH-3-OCH_3E_2							
17	2-OH-3-OCH_3E_1							
18	2-OHE_2-1-SG							
19	2-OHE_2-4-SG							
20	2-OHE_1-1-SG							
21	2-OHE_1-4-SG							
22	2-OHE_2-1 + 4-Cys							
23	2-OHE_1-1-Cys	**6.2**	0.97	21.61	**3.4**	0.93	20.58	0.0236
24	2-OHE_1-4-Cys							
25	2-OHE_2-1-NAcCys							
26	2-OHE_2-4-NAcCys							
27	2-OHE_1-1-NAcCys							
28	2-OHE_1-4-NAcCys							
29	4-OHE_2-2-SG							
30	4-OHE_1-2-SG							
31	4-OHE_2-2-Cys	**4.3**	0.44	18.73	**0.9**	0.13	2.22	0.0001
32	4-OHE_1-2-Cys							
33	4-OHE_2-2-NAcCys							
34	4-OHE_1-2-NAcCys							
35	4-OHE_2-1-N7Gua	**0.30**	0.00	1.55	**0.48**	0.13	24.97	0.0229
36	4-OHE_1-1-N7Gua							
37	4-OHE_2-1-N3Ade	**0.20**	0.00	2.08	**0.40**	0.17	1.32	0.0177
38	4-OHE_1-1-N3Ade							
39	2-OHE_2-6-N3Ade	**0.00**	0.00	1.79	**0.03**	0.00	0.24	0.754
40	2-OHE_1-6-N3Ade							

[a]The values reported in this table are the sum of the E_1 and E_2 forms of each metabolite, the sum of the thiol conjugates of 2-OHE_1(E_2), the sum of the thiol conjugates of 4-OHE_1(E_2) and the sum of the E_1 and E_2 forms of each depurinating DNA adduct. [b]p-Value comparing healthy control vs NHL by using the Mann–Whitney test.

UPLC-MS/MS conditions prior to analysis. Also for each batch, the standard mixture was run before the first sample, after the 5th sample and after the last (10th) sample to prevent errors due to matrix effect and day-to-day instrument variations. In addition, immediately after the initial standard and before the first sample, two spiked samples were run to calibrate for the drift in the retention time of all estrogen-related compounds due to the matrix effect. After UPLC analysis, the mean value was calculated for all the compounds detected in each sample.

Statistical analyses

After UPLC analysis, the median value and range were calculated for individual compounds obtained from each sample, which was analysed in triplicate. Differences in median values between controls and NHL cases were tested using the Mann–Whitney *U* statistic because the distributions were non-normal and not all of the distributions could be improved by transformation. Odds ratios (ORs) with 95% confidence intervals (CIs) were calculated to compare effect sizes between cases and controls for the ratio, after natural log transformation to improve normality. For all analyses, a two-sided *p*-value <0.05 was interpreted as a statistically significant difference and CIs were calculated at 95%. Analyses were conducted using GraphPad Prism software V 4.03 (GraphPad Software, La Jolla, CA, USA) and SAS (The SAS Institute, Cary, NC, USA).

Results

This study of the possible relationship between formation of estrogen-DNA adducts and NHL was conducted by analysing the estrogen metabolites, conjugates and depurinating DNA adducts in urine samples from 15 men diagnosed with NHL. Their disease characteristics, treatment and follow-up are presented in Table 1. The relative levels of the estrogen-DNA adducts in these samples were compared with those in urine samples from 30 age-matched healthy control men.

Following partial purification of the urine samples by SPE, we analysed the 40 estrogen-related compounds (Table 2). The values obtained from cases and healthy controls were analysed in two different ways. First, the median values were compared for all the compounds in the urine samples for the two groups of men (Table 3). Then, we calculated and compared the ratio of depurinating N3Ade and N7Gua adducts to the sum of their respective estrogen metabolites and conjugates (see footnote to Table 2) in urine samples from the two groups of men (Figure 3) because the ratio reflects the

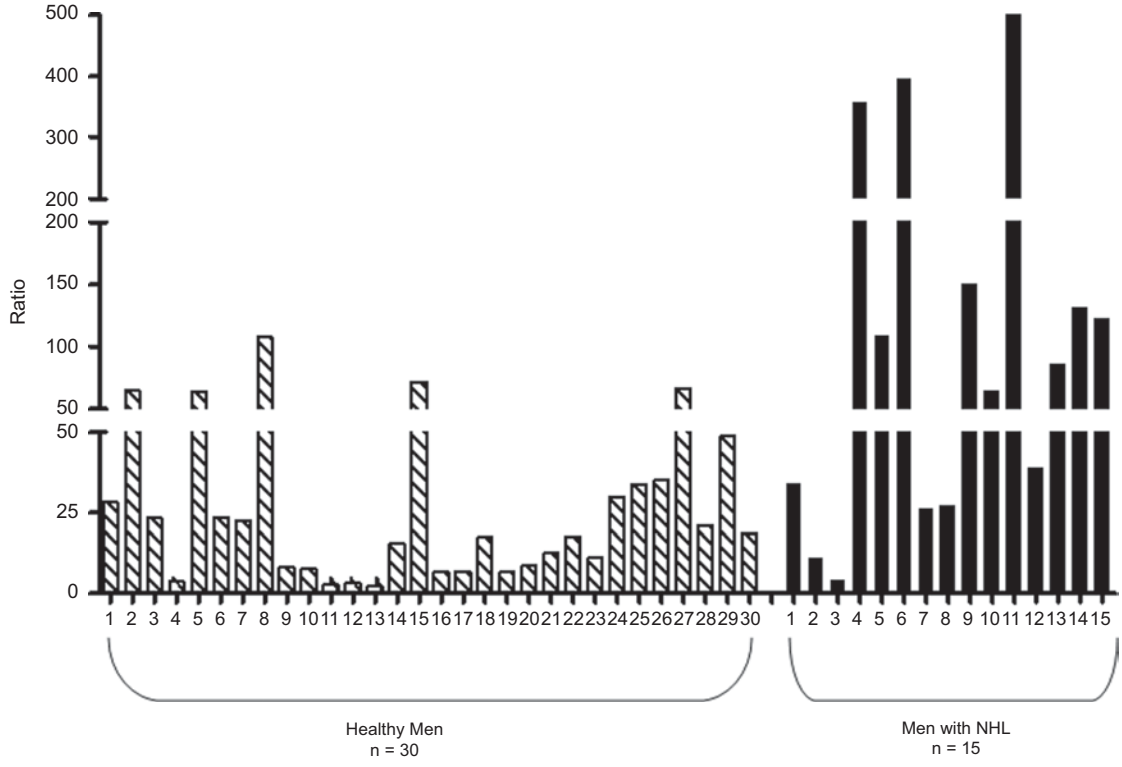

Figure 3. Individual ratios of depurinating estrogen-DNA adducts to estrogen metabolites and conjugates in urine of healthy control men and men with non-Hodgkin lymphoma (NHL). Healthy controls vs NHL, $p < 0.007$.

degree of imbalance in estrogen metabolism that can lead to cancer initiation.

Comparison of the median values of the urinary estrogen-related compounds from NHL cases and healthy controls indicated that the androstenedione and testosterone levels were higher (p <0.0001) in control men (Table 3). There were no differences in the median levels of E_1-sulfate, 2-$OHE_1(E_2)$ and 4-$OHE_1(E_2)$ between cases and controls, whereas 16α-$OHE_1(E_2)$ and the 2-$OHE_1(E_2)$-conjugates were found to be higher in the controls (Table 3). The median values of 4-$OCH_3E_1(E_2)$ (p <0.0087) and 4-$OHE_1(E_2)$-thiol conjugates (GSH, Cys, NAcCys) (p <0.0001) were significantly higher in the controls than in the cases (Figure 4). In contrast, the median levels of depurinating 4-$OHE_1(E_2)$-1-N7Gua (p <0.0229) and 4-$OHE_1(E_2)$-1-N3Ade (p <0.0177) adducts were higher in the cases than in the controls (Figure 5). No difference was found between the median levels of 2-$OHE_1(E_2)$-6-N3Ade adducts.

To find the balance between activating and deactivating pathways in both cases and controls, we further analysed the data using the ratio of depurinating 4-$OHE_1(E_2)$-1-N3Ade, 4-$OHE_1(E_2)$-1-N7Gua and 2-$OHE_1(E_2)$-6-N3Ade adducts to the sum of their respective estrogen metabolites and conjugates in urine samples as a continuous variable (see Table 2 footnote for calculating the ratio). In the sum of the ratios of depurinating adducts to estrogen metabolites and conjugates, the preponderant role is played by the N3Ade and N7Gua adducts of 4-$OHE_1(E_2)$, whereas the adducts of 2-$OHE_1(E_2)$ play a very minor role. The individual ratios of depurinating 4-$OHE_1(E_2)$-1-N3Ade and 4-$OHE_1(E_2)$-1-N7Gua adducts to the sum of their respective estrogen metabolites and conjugates in urine samples from men with NHL and healthy controls are presented in

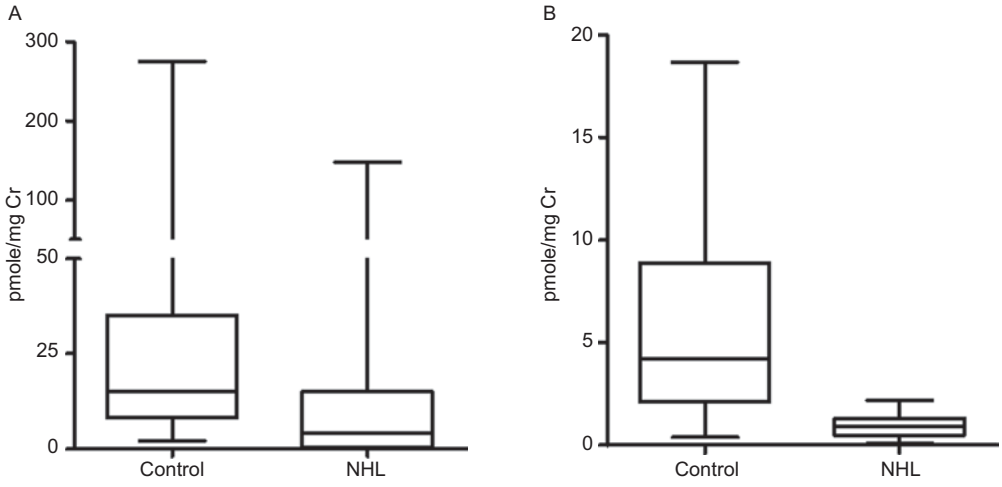

Figure 4. Urinary median levels of (A) 4-$OCH_3E_1(E_2)$ and (B) 4-$OHE_1(E_2)$ conjugates (GSH, Cys, NAcCys) from non-Hodgkin lymphoma (NHL) cases and controls.

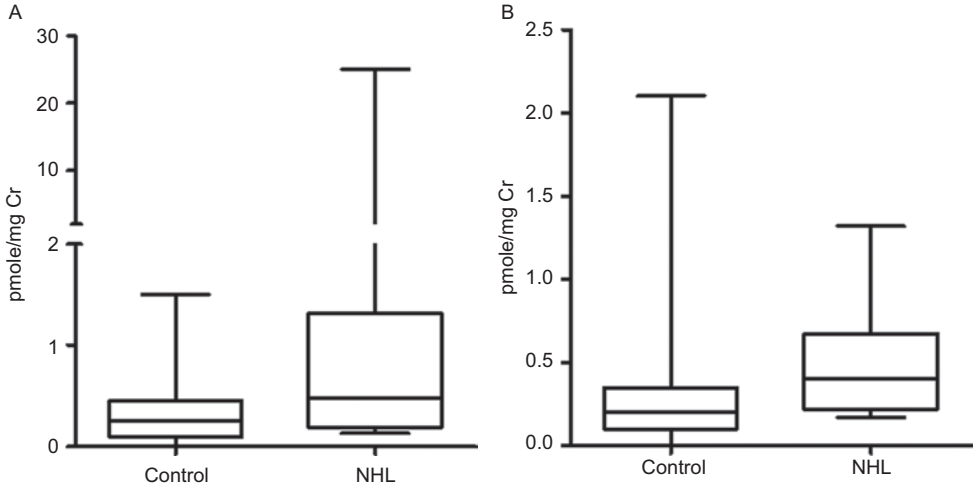

Figure 5. Urinary median levels of (A) 4-$OHE_1(E_2)$-1-N7Gua and (B) 4-$OHE_1(E_2)$-1-N3Ade adducts from non-Hodgkin lymphoma (NHL) cases and controls.

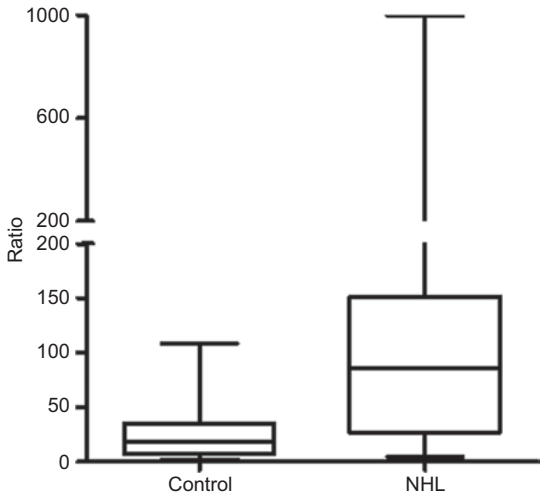

Figure 6. The ratio of depurinating DNA adducts to the sum of the corresponding estrogen metabolites and conjugates in non-Hodgkin lymphoma (NHL) cases and healthy controls. The NHL groups contained 15 subjects, and control group, 30 subjects. The statistical analysis by the Mann–Whitney test revealed that ratios for control men were significantly lower than those for the NHL men (p <0.0007).

Figure 3. Analysis using the Mann–Whitney *U* test revealed a significant difference between the two groups (p <0.0007) (Figure 6). The median relative level of estrogen-DNA adducts in the cases, 86.0, was significantly higher than the median level in the controls, 18.0, and having a high adduct ratio was associated with a nearly threefold increase in the odds of having NHL (OR 2.93; CI 1.41, 6.12).

Discussion

Cancer initiation by estrogens is based on estrogen metabolism that involves a disrupted homeostatic balance between activating and deactivating pathways (Gaikwad et al. 2008). Several factors can unbalance estrogen homeostasis, which is the equilibrium between estrogen activating and deactivating pathways that minimizes oxidation of catechol estrogens to quinones, and their subsequent reaction with DNA (Figure 2) (Cavalieri et al. 2006). A similar disruption in estrogen homeostasis has also been observed in men with prostate cancer (Yang et al. 2009) and women with breast cancer (Gaikwad et al. 2008, 2009a), as well as several animal models of estrogen carcinogenesis (Cavalieri et al. 2001, 2002a, Devanesan et al. 2001).

In the current study we examined urinary profiles of men with NHL and healthy controls. Comparison of the estrogen metabolic profiles of these two groups clearly shows that the median 4-$OCH_3E_1(E_2)$ and 4-$OHE_1(E_2)$-thiol conjugate (GSH, Cys, NAcCys) values were higher in healthy controls compared with NHL cases (Figure 4). In contrast, the median 4-$OHE_1(E_2)$-1-N3Ade and 4-$OHE_1(E_2)$-1-N7Gua values were lower in healthy controls compared with NHL cases (Figure 5). These results suggest that, in healthy control subjects, estrogen metabolism is balanced, i.e. the level of estrogen-DNA adducts is low and/or the levels of estrogen metabolites and conjugates are high. In contrast, in subjects with NHL, estrogen metabolism is unbalanced, i.e. the levels of depurinating estrogen-DNA adducts are high and/or the levels of estrogen metabolites and conjugates are low. Although the elevated adduct formation is tissue specific, we have demonstrated empirically in earlier studies of breast and prostate cancer patients that these adduct biomarkers are excreted in the urine at higher concentrations (Gaikwad et al. 2008, 2009a, Yang et al. 2009).

Observation of high levels of depurinating estrogen-DNA adducts in urine from men with NHL (Figure 5) is consistent with the hypothesis that these adducts may be important in the aetiology of NHL and serve as biomarkers of risk. The median 2-$OHE_1(E_2)$, 2-$OCH_3E_1(E_2)$ and 2-$OHE_1(E_2)$-6-N3Ade values in both groups did not show any trend, indicating that the 2-catechol pathway is not important in the aetiology of NHL. Furthermore, the adduct to metabolite and conjugate ratios were analysed to quantify the degree of imbalance in the estrogen metabolism. The ratios obtained for men with NHL were found to be higher, whereas the ratios found in the healthy control men were lower. The higher ratios reflect the relatively higher levels of DNA adducts in the urine of men with NHL, and suggest that high levels of estrogen-DNA adducts may be an important factor in the initiation of NHL. In fact, despite the different expression of NHL seen in the subject population (Table 1), the first step, reaction of estrogen quinone with DNA, in the initiation of NHL is hypothesized to be the same. However, further, more extensive studies of these biomarkers are needed to confirm these findings.

The OR observed here of 2.93 (CI 1.41, 6.12) with a sample size of 45 is similar to the effects size of 3.07 (CI 1.50, 6.26) seen previously in a study of men with (n = 14) and without (n = 125) prostate cancer (Yang et al. 2009), but is somewhat lower than that observed in women with (n = 40) and without (n = 40) breast cancer (OR 4.88; CI 2.30, 10.4) (Gaikwad et al. 2009a). Power calculations based on these NHL data showed sufficient sample sizes to detect a twofold difference between cases and controls of the 4-$OHE_1(E_2)$-1-N7Gua adduct levels (>95%), but much less power to detect differences in 4-$OHE_1(E_2)$-1-N3Ade. However, we had nearly 90% power to detect a twofold difference in the adduct ratio between NHL cases and controls in this study, an estimate that has been consistent in power calculations in previous data of breast and prostate cancer studies. The consistency of results across these cancer studies implicates estrogen imbalance as playing a causative role, as

depicted in Figure 2, but future studies should replicate these findings in a larger population.

In conclusion, men with NHL had relatively high levels of estrogen-DNA adducts in their urine. Our results support the hypothesis that formation of estrogen-DNA adducts could be an important event in the initiation of NHL. Hence, these estrogen-DNA adducts should be evaluated as biomarkers for NHL risk in future studies. In addition, these adducts could be used to investigate the ability of putative preventive compounds to reduce the risk of developing NHL. Based on this study, one could speculate that minimizing the formation of catechol estrogen quinones and/or their reaction with DNA (Gaikwad et al. 2007, 2009b, Zahid et al. 2007, 2008, Venugopal et al. 2008, Lu et al. 2008) should reduce the risk of developing NHL. These preventive effects have been seen *in vitro* (Zahid et al. 2007) and in cell cultures (Venugopal et al. 2008, Lu et al. 2008, Zahid et al. 2008) treated with specific putative preventive compounds.

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