Hb concentrations. Standard techniques commonly used for screening for Hb variants, such as electrophoresis, IEF, or ion-exchange HPLC, are not sufficient because 33 of the 89 different Hb variants associated with erythrocytosis do not induce any major change in net charge of the Hb molecule. The additional use of ESMS is encouraged because combination of this technique and IEF will increase the sensitivity for Hb variants that cause erythrocytosis to 100%.

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Quantification of Aromatic Hydrocarbon Receptor (*AHR*) and Related Genes by Calibrated Reverse Transcription-PCR in Blood Mononuclear Cells, *Cristina Dassi,[1] Paolo Brambilla,[2] Stefano Signorini,[1] Piermario Gerthoux,[1] Pierangela Molteni,[1] Raffaella Sala,[1] and Paolo Mocarelli[1*]* ([1] University Department of Laboratory Medicine, University of Milano-Bicocca, Hospital of Desio, Via Mazzini 1, 20033 Desio, Milan, Italy; [2] Department of Laboratory Medicine, Hospital of Merate, Largo Mandic 1, 23807 Lecco, Italy; * author for correspondence: fax 39-036-2383464, e-mail mocarelli@uds.unimb.it)

The aromatic hydrocarbon (*Ah*) gene battery *(1)* comprises Ah receptor (*AHR*), its nuclear translocator (*ARNT*), and the genes that encode the detoxifying enzymes of phase I (e.g., cytochrome P4501A1, -1A2, and -1B1) and phase II. These enzymes and growth regulatory factors such as interleukin 1-β (IL-1β) are coordinately induced by polychlorinated dibenzo-*p*-dioxins and polycyclic aromatic hydrocarbons, toxic chemicals that are ubiquitous in ambient air and in cigarette smoke, via AHR *(1–4)*. Quantification of the regulation of *AHR* and related genes could be useful to monitor the degree of polycyclic aromatic hydrocarbon exposure and to understand the pathways to chemical carcinogenesis and atherosclerosis *(5)*. The expression of *AHR* and related genes has been studied using Northern blot analysis *(6)* or quantitative reverse transcription-PCR (RT-PCR) using labeled primers *(7)*, which are low-sensitivity techniques; competitive RT-PCR *(8)*, which is more sensitive but quite laborious; and real-time PCR *(9)*, which is expensive.

An interesting technique, proposed for the first time by Tsai and Wiltbank *(10)* and considered later by Bor et al. *(11)*, is called "calibrated " RT-PCR; it is a competitive RT-PCR based on the addition of a fixed amount of internal standard (IS) to the sample and to a set of calibrators. The labor, time, and costs for this method are lower than those for classic competitive RT-PCR. We present calibrated RT-PCR assays to study the expression of *AHR*, *ARNT*, cytochrome P4501B1 (*CYP1B1*), and *IL-1*β for the *AHR* gene battery and of β-*actin* (β-*ACT*) as housekeeping gene in peripheral blood mononuclear cells from 32 healthy subjects.

Total RNA was extracted according to the method of Chomczynski and Sacchi *(12)* from 1×10^7 peripheral blood mononuclear cells in suspension isolated by step-gradient centrifugation on Histopaque. RNA was resuspended in 50 μL of sterile water, quantified by measuring the absorbance at 260 nm, diluted to 100 mg/L with sterile water, and stored in liquid nitrogen.

The ISs, designed to be smaller than the corresponding target sequences, were homologous recombinant cDNAs obtained by oligonucleotide-mediated mutagenesis *(13)* from *AHR*, *ARNT*, *CYP1B1*, and β-*ACT* sequences inserted in pGEM plasmid. A more recently developed and simple method *(14, 15)* was adopted to obtain the *IL-1*β IS. Table 1 shows the primers used for the amplification and the oligonucleotides used to produce corresponding ISs. Forward and reverse primers were chosen on differ-

ent exons to include the intervening sequences when the DNA was amplified. Plasmids containing ISs were linearized and transcribed into RNA using the RiboMAX™ Large Scale Production System T7 (Promega) followed by an RNase-free DNase treatment to remove the DNA template. ISs were purified according to the technical manual of RiboMAX™. RNA was diluted to the desired concentrations in sterile water. Ribosomal RNA 16S–23S from *Escherichia coli* was added (final rRNA concentration, 100 mg/L) and stored in liquid nitrogen. The rRNA from *E. coli* did not interfere with RT-PCR.

Competitive RT-PCR was performed as described previously *(16)*. Gene-specific PCR reagents and conditions were as follows: (*a*) $MgCl_2$, 1.5 mM for *ARNT* and *CYP1B1* and 2 mM for *AHR*, *IL-1β*, and *β-ACT*; (*b*) Taq polymerase, 1.25 U for *AHR*, *ARNT*, and *CYP1B1* and 2.5 U for *IL-1β* and *β-ACT*; (*c*) annealing-extension temperature, 59 °C for *IL-1β*, 60 °C for *β-ACT* and *AHR*, 63 °C for *ARNT* and *CYP1B1;* (*d*) cycles numbers, 33 for *AHR*, 35 for *ARNT*, *CYP1B1*, and *β-ACT*, 37 for *IL-1β*. Quantification was performed as described previously *(16, 17)*, with a correction factor of 1.28 for *AHR*, 1.2 for *ARNT* and *IL-1β*, 1.19 for *CYP1B1*, and 1.24 for *β-ACT*, accounting for the difference in size of the target:IS ratio.

Calibrated RT-PCR was performed according to Bor et al. *(11)* by adding a fixed amount of IS to the unknown samples and to a set of calibrators containing 0.35, 0.67, 6.03, 0.097, and 2939 amol of mRNA in 260 ng of total RNA, respectively, for *AHR*, *ARNT*, *CYP1B1*, *IL-1β*, and *β-ACT*. Serial dilutions were prepared from each first calibrator to obtain calibrators with lower concentrations (220, 180, 140, 100, 50, 10, and 5 ng of total RNA). A calibration curve was constructed by plotting the ratio, calibrator mRNA:RNA IS, against the amount of calibrator mRNA. The amplified target and the IS were separated by electrophoresis. The mRNA concentrations in the unknown samples were calculated from the calibration curve based on the ratio between the sample and the corresponding RNA IS. The reverse transcription and PCR conditions used were the same as those for the competitive RT-PCR.

Quantification of calibrated RT-PCR was performed as described previously *(11)*, using the above-mentioned correction factors.

The target:IS ratio results were independent of the number of cycles (25–40) of amplification, indicating that, for all genes, the relative amounts of the two amplified products stayed identical throughout the PCR amplification exponential phase and when the plateau phase was reached.

An example of an agarose gel showing the calibration curve for calibrated RT-PCR of *AHR* performed with a constant amount of RNA IS (0.166 amol) is shown in Fig. 1A. The calibration curve is linearly described by the equation $y = ax + b$, where x is the calibrator total RNA and y represents the ratio of the target product to the IS product (Fig. 1B). This equation was determined for each assay and used to detect the amount of sample mRNA. One sample for *AHR* and 10 samples for *IL-1β* exceeded the calibration curve and were tested using calibration curves covering a higher linear range (up to 500 ng of total RNA for *AHR* and up to 1000 ng for *IL-1β*). We preferred to use a higher concentration of IS instead of diluting the sample to maintain the characteristics of the sample.

The calibrated RT-PCR showed good intraassay imprecision; the CV (calculated from 10 replicates of a sample) was 7.3%, 6.8%, 8.7%, 6.0%, and 6.9% for *AHR*, *ARNT*, *CYP1B1*, *IL-1β*, and *β-ACT*, respectively. Interassay imprecision was calculated from results of duplicate RT-PCRs in two different analytical runs on the same RNA preparation of 32 subjects; the CV was 10%, 8.5%, 11%, 8.0%, and 8.0% for *AHR*, *ARNT*, *CYP1B1*, *IL-1β*, and *β-ACT*, respectively. These results are similar to values reported previously *(11)*.

The calibrated RT-PCR is suitable for a wide range of measurements; it therefore can be used to quantify genes

Table 1. Primers used for RT-PCR and IS preparation.

Name	Primer	Sequence (5′-3′)	RT-PCR product, bp
AHR	Forward	gtgcttcatatgtcgtctaag	915, sample 714, IS
	Reverse	aatgagttcacatcctgaggc	
	Oligonucleotide	actgaagcagagctgcagagaccactaacag	
ARNT	Forward	cggaacaagatgacagcctac	520, sample 430, IS
	Reverse	acagaaagccatctgctgcc	
	Oligonucleotide	ggtacccacctgtagggcagtttctcacatg	
CYP1B1	Forward	gtgatgccctggctgcag	608, sample 510, IS
	Reverse	aatcgagctggatcaaagttc	
	Oligonucleotide	cggccactatcactggaattggatcaggtc	
IL-1β	Forward	gcatccagctacgaatctcc	318, sample 265, IS
	Reverse	ccatatcctgtccctggag	
	Oligonucleotide	gcatccagctacgaatctccggacaagctgaggaa	
β-ACT	Forward	gtgcgtgacattaaggagaag	213, sample 171, IS
	Reverse	gaaggtagtttcgtggatgc	
	Oligonucleotide	gagaagagctacgagcgctgccctgaggcac	

whose expression can vary widely in different subjects. The expression of *AHR* and related genes, expressed as amol mRNA/100 ng of total RNA, was 0.012–0.55 for *AHR*, 0.07–0.36 for *ARNT*, 0.02–6.14 for *CYP1B1*, 0.015–0.33 for *IL-1β*, and 378-1940 for *β-ACT*. A higher sensitivity can be obtained by increasing the number of PCR cycles, as we did with the *IL-1β* gene, whose expression was lower respect to the other investigated genes, but keeping in mind that heteroduplex formation can occur with an excessive number of cycles.

Samples from 30 different subjects were analyzed with both competitive RT-PCR and calibrated RT-PCR assays for all genes tested. Weighted Deming regression analysis was performed on all data, and the regression coefficient (R), slope (95% confidence interval), and y-intercept (95% confidence interval) for each gene was as follows: for *AHR*, $R = 0.951$, slope = 0.90 (0.7–1.05), y-intercept = 0.05 amol (−1.22 to 1.32 amol); for *ARNT*, $R = 0.912$, slope = 1.0 (0.7–1.3), y-intercept = −2.09 amol (−6.1 to 1.9 amol); for *CYP1B1*, $R = 0.948$, slope = 1.14 (0.9–1.4), y-intercept = −0.22 amol (−2.19 to 1.73 amol); for *IL-1β*, $R = 0.967$, slope = 1.05 (0.9–1.2), y-intercept = 0.1 amol (−0.53 to 0.73 amol); for *β-ACT*, $R = 0.961$, slope = 1.1 (0.9–1.3), y-intercept = −0.37 amol (−1.0 to 0.28 amol).

The results obtained with calibrated RT-PCR were in agreement with those obtained with classic competitive RT-PCR for all tested genes, showing a good correlation between the two techniques.

Compared with the competitive RT-PCR, calibrated RT-PCR has the advantage of requiring a smaller amount of biological sample (one replicated tube instead of six single tubes), which is important when the amount of the sample is a limiting factor, and allows reduced labor, time, and costs. The amount of mRNA for the target genes is strictly dependent on the corresponding RNA IS preparation, a procedure that has an intrinsic variability associated with in vitro transcription, purification from nucleotide, and quantification. Different from the method of Bor et al. *(11)*, where the IS and target RNA share only the sequences of the primer sites, our synthetic RNA has a sequence identical to that of the target mRNA except the deleted region so that there are no differences in amplification efficiencies; the difference in size between mimic and target allows separation of the corresponding amplification products in an agarose gel. We preferred to clone the IS fragments into a vector to facilitate their spectrophotometric quantification, as reported by Tsai and Wiltbank *(10)*. False-positive results attributable to the amplification of DNA potentially present in samples after the RNA extraction process were avoided by an appropriate choice of primer sequences in two different exons.

To minimize variability, it is advisable to prepare a sufficient quantity of RNA IS that can be used for analyses over a longer period. Our RNA IS, added to a larger amount (100 mg/L) of ribosomal RNA to prevent RNA degradation and stored in liquid nitrogen, showed good stability over time. Quantification with this technique can potentially be applied to study gene expression in every tissue that allows RNA extraction.

In conclusion, calibrated RT-PCR is a reliable and accurate method for evaluating the expression of *AHR* and a related battery of genes that have been proposed as mediators of the majority of biochemical and toxicologic effects of several xenobiotics.

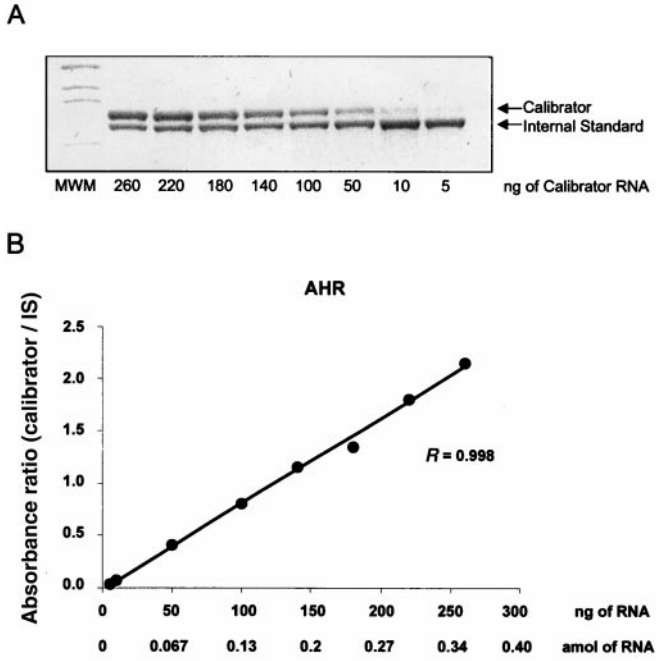

Fig. 1. Gel showing calibration of *AHR* (*A*) and plot of the ratio of the target product to the IS product plotted against the amount of calibrator RNA (*B*).

(*A*), ethidium bromide-stained agarose gel from calibrated RT-PCR for *AHR* performed with a constant amount of RNA IS (0.166 amol). The 915-bp bands correspond to *AHR* mRNA and the 667-bp bands to the synthetic IS. *Lane MWM*, molecular weight markers.

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Biomarkers of Bone Turnover after a Short Period of Steroid Therapy in Elderly Men, *Federica Paglia,[1] Simona Dionisi,[1] Simona De Geronimo,[1] Rossana Rosso,[1] Elisabetta Romagnoli,[2] Natalia Raejentroph,[1] Alessandro Ragno,[1] Massimiliano Celi,[1] Jessica Pepe,[1] Emilio D'Erasmo,[1] and Salvatore Minisola[1*]* ([1] Dipartimento di Scienze Cliniche, Università di Roma "La Sapienza", 00161 Rome, Italy; [2] Ospedale "Casa Sollievo della Sofferenza" IRCCS, San Giovanni 71013 Rotondo (FG), Italy; address correspondence to this author at: Department of Clinical Sciences, Policlinico Umberto I, Viale del Policlinico 155, 00161 Rome, Italy; fax 39-06-68803566, e-mail salvatore.minisola@uniroma1.it)

An excess of glucocorticoids is the third most common cause of bone loss after postmenopausal and senile osteoporosis. The iatrogenic form of glucocorticoid-induced osteoporosis, which is more frequent than Cushing syndrome, has uncertain cellular and molecular bases. The lack of consistent information concerning the pathophysiology of corticosteroid-related bone loss is attributable to several coexisting factors, e.g., the heterogeneity of the underlying disease, that may themselves influence skeletal loss, dosage, and duration of treatment *(1, 2)*.

A well-known action of steroids on bone is decreased bone formation. There are no consistent findings concerning bone resorption in glucocorticoid-induced osteoporosis. The aim of this study was to evaluate the impact of a short period of steroid therapy on the serum concentrations of bone biomarkers in elderly men.

We studied 14 elderly men (mean age ± SD, 66.1 ± 6.4 years; range, 57–76 years), admitted to our hospital from October 1998 to June 1999, who were suffering from various medical pathologies requiring systemic steroid therapy. Four patients were suffering from pulmonary diseases, four from immunologic, two from cerebral, and four from neoplastic diseases without bone metastases. None of these patients was bedridden. The patients were studied while undergoing treatment, which lasted no more than 30 days (mean ± SD, 9.1 ± 9.6 days), with a cumulative dose of 10–1250 mg of prednisone (mean prednisone equivalent, 338 ± 382 mg) or its equivalent. The patients were compared with 14 hospitalized patients of similar age (67.9 ± 6.7 years) without any history of bone illness who were not taking drugs known to affect bone tissue. The mean period of hospitalization was similar for patients and controls at the time of investigation. Informed consent was obtained from patients and controls to perform this investigation.

Metabolic tests included a blood sample, collected in the morning between 0800 and 1000, to evaluate serum biomarkers of bone turnover.

Bone resorption was assessed by measuring tartrate-resistant acid phosphatase activity (TRAP) and the C-terminal telopeptides of type I collagen (βCTx; Osteometer Biotech A/S), whereas bone formation was assessed by measuring serum osteocalcin (BGP; N-tact Osteo SP; Incstar Co.) and bone-specific alkaline phosphatase (ALP; Alkphase B; Metra Biosystem Inc.). Serum TRAP was assayed by a spectrophotometric assay used in our laboratory *(3)*. Serum βCTx was measured by a two-site ELISA based on two highly specific monoclonal antibodies against the amino acid sequence AHD-β-GGR, where the aspartic acid residue (D) is β-isomerized *(4)*. The detection limit, defined as the concentration corresponding to 2 SD above the mean of 15 determinations of the zero calibrator, was 75 pmol/L. We assessed imprecision (as CV) by measuring three serum samples in eight consecutive analytical runs (5%) and each of the three samples in the same analytical run (9.8%). Serum bone ALP was assayed by an immunoenzymatic assay and serum BGP by an immunoradiometric assay used in our laboratory. The details of these two methods are reported elsewhere *(5, 6)*.

We found no difference between patients and controls for mean serum TRAP (10.5 ± 2.4 vs 10.6 ± 2.3 U/L), whereas mean serum βCTx was significantly higher in steroid-treated patients compared with controls (5194 ± 2617 vs 1491 ± 774 pmol/L; $P < 0.001$; Fig. 1). Bone ALP was similar between the two groups (17.1 ± 4.5 vs 18.1 ± 3.6 U/L), whereas we found a statistically significant difference for serum BGP (1.4 ± 1.1 vs 5.1 ± 1.4 μg/L; $P < 0.001$; Fig. 1). Finally, serum BGP was inversely correlated with the cumulative dose of steroids ($r = -0.56$; $P < 0.05$).

During glucocorticoid treatment, there is biphasic bone loss with a rapid initial loss of ~12% during the first few months, followed by a slower phase of ~2–5% annually *(1)*. The effect of glucocorticoids on bone consists of reduced bone formation with decreased wall thickness of the trabeculae, a strong indication of the decreased output of osteoblasts *(7, 8)*. In agreement with these data, our