190 spin column (PreAnalytiX; PreAnalytic); resulting in selective binding of
191 RNA to the silica-gel membrane of the spin column. After the column was
192 washed three times, pure RNA was eluted in 80 μL of RNase-free water.
193

194 ***Real-time quantitative RT-PCR***

195 Reverse transcription of the mRNA was performed using an
196 Omniscript RT Kit (Qiagen, Hilden, Germany). Real-time quantitative PCR
197 was then performed using a QuantiTect Probe PCR Kit (Qiagen). RT-PCR
198 was performed according to the manufacturer's instructions. cDNA products
199 were amplified by real-time quantitative PCR according to the
200 manufacturer's instructions (QuantiTect Probe PCR kit, Qiagen) using a 2
201 μL aliquot of cDNA and the kit's components in a reaction volume of 20 μL.
202 TaqMan PCR analyses for VEGF, Flt-1, ENG、PlGF, TGF-β1, P-Selectin,
203 Placenta specific-1 (PLAC-1), HO-1 and SOD were performed using
204 predeveloped and commercially available primers and probe sets (Cat #
205 Hs00900054_m1 for VEGF, Cat # Hs01052936_m1 for Flt-1, Cat #
206 Hs00923997_g1 for ENG, Cat # Hs00182176_m1 for PlGF, Cat #
207 Hs0000171257_m1 for TGF-β1, Cat # Hs00174583_m1 for P-Selectin,

208 TaqMan Probes for PLAC-1 gene have been described previously[21], Cat #
209 Hs00157965_m1 for HO-1, and Cat# Hs00166575_m1 for SOD; Applied
210 Biosystems, Foster City, CA). The thermal cycling protocol used for PCR
211 was: initial denaturation at 95°C for 15 min, 40 cycles of denaturation at
212 94°C for 15 sec, and annealing at 60°C for 1 min. Initially each PCR assay
213 was confirmed to be specific to mRNA and not to genomic DNA.
214 Amplification data were collected and analysed with an ABI Prism 7900T
215 Sequence Detector (Applied Biosystems). Each sample was analysed in
216 duplicate, and multiple negative water blanks were included in every
217 analysis. Quantification of gene expression was performed by investigators
218 blinded to the outcome of the pregnancy. The amounts of mRNA samples
219 were expressed in term of copies per millilitre by the method reported
220 elsewhere [22].
221
222 ***Statistical analysis***
223 The distributions of the demographic characteristics and Log mRNA
224 concentrations were analyzed after conversion to multiples of the median
225 (MoM). MoMs were also adjusted for the body mass index. Median values

226 were retrospectively stratified according to the severity of PE and the
227 development of HELLP syndrome. The non parametric analysis of variance
228 (Kruskal Wallis test) and Dunn post hoc test were used for comparisons
229 among and between groups. The sensitivity and 1-specificity rate (false
230 positive rate) was calculated for each available marker using a univariate
231 receiver operating characteristic (ROC) curve. A multivariate analysis was
232 performed using logistic regression to calculate the odds for each patient for
233 classification as a case. The logistic output was adjusted for the incidence of
234 PE in the general population (2%) by calculation of the sampling fraction as
235 described by Collett[25]. Finally, a ROC curve for the calculation of
236 multivariable sensitivity was built using, as the test variable, the calculated
237 odds for PE by a logistic regression analysis for each patient in the series.
238

239 **RESULTS**

240 The demographics of the pregnant women from control and PE groups
241 are shown in **Table 1**. Although no differences were observed in the
242 maternal age, body mass index (BMI), gestational age at blood drawing, and
243 blood pressure at the time of blood drawing between groups, significant

244 differences were noted in birth weight and gestational age at delivery, and
245 these findings were consistent with those reported in the existing literature.
246 The values of the available markers are reported in **Figure 1a-g**.
247 The mRNA expression levels of Flt-1, ENG, P-Selectin and PLAC-1 were
248 higher in PE than in controls, but PlGF and HO-1 was lower. TGF-β1, VEGF
249 and SOD did not reach a significant value in the comparison of PE cases vs.
250 controls, (Kruskal-Wallis test) and were therefore excluded from any further
251 analysis. For all the other markers a significant difference was found in the
252 comparisons of HELLP cases vs. controls and for some of them including
253 Flt-1, ENG, PlGF a significant difference was found for any generated
254 subgroup (mild PE, severe PE, HELLP) according with the severity (Dunn
255 post hoc test). An univariate analysis identified Flt-1 and ENG as the
256 markers with the highest sensitivity. **Table 2** reports the output of the ROC
257 curves analysis for those markers with a significant difference in PE cases vs.
258 controls along with the actual MoM values for each marker associated with
259 that given sensitivity. The best multivariable model was obtained by
260 combining Flt-1, ENG, PlGF and parity **(Table 3)**. Only the final model has
261 been presented which was obtained by stepwise logistic regression. After

262 adjusting for the incidence of PE (2% or 1:50), the mean quoted odds
263 (obtained by solving the logistic equation having the odds as a dependent
264 variable for each of the subjects in the study) for PE was 1:50 (0.02) for
265 controls and 1:7 (0.14) for subjects who developed PE. A ROC curve obtained
266 using the estimated score for PE as the test variable yielded a sensitivity of
267 66% at a 10% 1-specificity rate with an area under the curve of 0.884
268 (0.844-0.922, 95% CI; $p<0.001$; **Figure 2**) therefore with a reasonable
269 discrimination to identify cases. Again, sensitivity and the odds for each
270 patient for classification as PE were correlated with the severity
271 (sensitivity= 50% for mild PE, 70% for severe PE and 75% for HELLP;
272 odds=1:12 for mild PE, 1:5 for both severe PE and HELLP).
273
274 **COMMENTS**
275 The present study prospectively examined the mRNA expression in
276 the cellular component of maternal blood samples from gestational week
277 15-20. The study assessed nine species of gene expression associated with
278 angiogenesis and oxidative stress, which coded for factors thought to be
279 important in the pathogenesis of PE. The mRNA levels were compared with

280 the clinical outcomes. As a result, the mRNA expression levels of ENG, Flt-1,
281 P-selectin and PLAC-1 were significantly higher in PE than those of control,
282 whereas PlGF and HO-1 was lower in PE. The blood samples were obtained
283 at an average of 17 weeks, which is almost 20 weeks before the onset of PE.
284 At this gestational age, mRNA expression of anti-angiogenic factors and
285 anti-oxidants are already altered in pregnant women who subsequently
286 develop PE. These factors have a crucial role in the pathogenesis of PE, and
287 the analysis of cellular components of maternal blood for these transcripts
288 may allow for the prediction of PE.

289 Although many tests have been proposed for the prediction of PE,
290 results have been inconsistent and contradictory [26-28]. The present study
291 proved that a panel of cellular RNA quantified long before clinical onset
292 predicted PE occurrence with a degree of accuracy comparable to previous
293 reports including uterine artery Doppler velocimetry with or without
294 demographical and biochemical parameters [26]. The univariate analysis
295 showed Flt-1 and ENG as the markers with the highest sensitivity. The best
296 multivariate model was obtained by combining Flt-1, ENG, PlGF and parity.
297 The ROC curve yielded a sensitivity of 66% at 10% 1-specificity rate with an

298 area under the curve of 0.884. These results indicated that cellular RNA in
299 maternal blood could be used to assess the pathophysiological alterations
300 which have occurred in pregnant women who later develop PE.

301 A previous study assessed the cell-free RNA in maternal plasma. The
302 expression of 7 transcripts was assessed in the plasma of pregnant women
303 of gestational week 15-20 [20]. The target genes were Flt-1, ENG, VEGF,
304 plasminogen activator inhibitor-1 (PAI-1), tissue-type plasminogen activator
305 (tPA), PLAC-1, and P-selectin. In the statistical univariate analysis, Flt-1
306 showed the highest degree of discrimination, followed by ENG, PAI-1,
307 P-selectin, tPA, VEGF and PLAC-1. The best multivariate model was
308 obtained by the combination of all markers. A ROC curve yielded a
309 sensitivity of 84% (95%CI 71.8-91.5) at a 5% 1-specificity rate with an area
310 under the curve of 0.927 ($p<0.001$) [20]. It has been previously suggested that
311 the cellular RNA analysis is a little debased in comparison to plasma RNA
312 analysis[17]. In our previous study of human placental lactogen gene, the
313 coefficient of variation of our cellular RNA analysis was approximately 20%;
314 it was not calculated in the current study and this is a significant limitation
315 of this study[17]. However, since the cellular RNA can be preserved in the

316 PAXgene Blood RNA tube, we have confirmed that the RNA was stable
317 under -20°C at least for a year. And the blood processing of cellular RNA is
318 much easier than plasma RNA. In addition, the expression levels of hPL or
319 hCG are approximately 10-times higher than those in plasma RNA[17]. We
320 therefore suggest that the analysis of plasma cellular RNA is a promising
321 method to evaluate the pathophysiological alterations occurring in pregnant
322 women who later develop PE. In both the previous cell-free RNA study and
323 the present cellular RNA study, the levels of Flt-1 and ENG were two of the
324 best predictors for PE. This fact indicated that these anti-angiogenic factors
325 have a crucial role in the pathogenesis of PE.

326 The origin of the cellular RNA seen in maternal blood has not been
327 resolved. In our previous study, hPL and hCG expression levels in the
328 cellular components of maternal blood correlated with the corresponding
329 protein levels[17,24]. This finding suggested that some trophoblasts or
330 placental debris circulate in the maternal blood. The half-times of the hPL
331 expressions in the cellular and plasma RNA were 203.8 and 32.2 min,
332 respectively. Therefore the half-time of the cellular RNA was much longer
333 than that of plasma RNA, and the cellular RNA was not removed from the

334 maternal circulation rapidly after delivery. The RNA originating from
335 circulating trophoblasts in maternal blood could be detected for several
336 months, as fetal nucleated erythrocytes reportedly circulate in maternal
337 blood for 3 months after delivery [29]. These findings suggest that the levels of
338 trophoblasts-derived RNA in the cellular RNA in maternal blood could
339 reflect the pathophysiological alterations of the placenta. However, real-time
340 evaluation of placental function through cellular RNA may be inferior to the
341 cell-free RNA.

342 Another study assessed the mRNA expression of trophoblasts
343 obtained from chorionic villous sampling (CVS) at week 11. The expression
344 levels of Flt-1, ENG, and VEGF in CVS tissue obtained from pregnant
345 women who later developed PE were higher and those of PlGF and HO-1
346 were lower than those of normal pregnancies [16]. These findings indicated
347 that the upregulation of anti-angiogenic factors and down-regulation of
348 anti-oxidant factors has already occurred in 1st trimester trophoblasts[16] and
349 that the alterations could be evaluated by the analysis of cellular RNA. All
350 of these findings support the hypothesis that some mRNA expressions of
351 Flt-1, ENG, PlGF, VEGF, and HO-1 are derived from circulating

352 trophoblasts, and that the alteration of these mRNA levels may reflect
353 mRNA alterations which are associated with the pathogenesis of PE in the
354 placenta. Therefore, it is suggested that evaluation of cellular mRNA may
355 allow for the indirect monitoring of the placental function.

356 In this study, PE occurred in 9% of pregnant women, a higher frequency
357 than seen in the published literature. No previous large studies have
358 clarified the prevalence of PE in Indonesian populations, and this higher
359 prevalence could confound the result. Furthermore, because the case number
360 of gestational hypertension or early onset type PE is not sufficient to analyze
361 statistically, the patients with hypertension in pregnancy were excluded and
362 those with early onset of PE were not analyzed separately. Further study in
363 more developed countries is needed to confirm the predictive efficiency of
364 cellular RNA in maternal blood.

365 In conclusion, the current study demonstrated that Flt-1 and ENG
366 expression increased in the cellular RNA in the blood from pregnant women
367 who developed PE, whereas HO-1 and PlGF expression decreased. These
368 alterations increased with the severity of clinical symptoms of PE at later
369 gestation. Furthermore, an analysis of the expression of these transcripts

370 allows accurate detection of high-risk pregnant women who are likely to
371 develop PE in populations at low risk for developing PE.
372
373
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375
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377
378 **Contribution to authorship:**
379 A. Sekizawa, T. Okai, Y. Purwosunu and A. Farina designed the
380 research and approved the final, submitted version. A. Sekizawa, M.
381 Nakamura, H. Shimizu, N. Wibowo, and Y. Purwosunu collected, analyzed,
382 interpreted data and drafted the manuscript. A. Farina and N. Rizzo
383 performed statistical analysis.
384
385 **Details of ethics approval:**
386 Approved by the ethics committee of Showa University, #86, and by
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396 **REFERENCES**

397 1. Redman CW, Sargent IL. Latest advances in understanding preeclampsia.
398 *Science* 2005;**308**(5728):1592-4.
399 2. Levine RJ, Lam C, Qian C, et al. Soluble endoglin and other circulating
400 antiangiogenic factors in preeclampsia. *N Engl J Med*
401 2006;**355**(10):992-1005.
402 3. Levine RJ, Maynard SE, Qian C, et al. Circulating angiogenic factors and the risk
403 of preeclampsia. *N Engl J Med* 2004;**350**(7):672-83.
404 4. Maynard SE, Min JY, Merchan J, et al. Excess placental soluble fms-like tyrosine
405 kinase 1 (sFlt1) may contribute to endothelial dysfunction, hypertension,
406 and proteinuria in preeclampsia. *J Clin Invest* 2003;**111**(5):649-58.
407 5. Venkatesha S, Toporsian M, Lam C, et al. Soluble endoglin contributes to the
408 pathogenesis of preeclampsia. *Nat Med* 2006;**12**(6):642-9.
409 6. Ferrara N. Role of vascular endothelial growth factor in regulation of
410 physiological angiogenesis. *Am J Physiol Cell Physiol* 2001;**280**(6):C1358-66.
411 7. He H, Venema VJ, Gu X, Venema RC, Marrero MB, Caldwell RB. Vascular
412 endothelial growth factor signals endothelial cell production of nitric oxide
413 and prostacyclin through flk-1/KDR activation of c-Src. *J Biol Chem*
414 1999;**274**(35):25130-5.
415 8. He Y, Smith SK, Day KA, Clark DE, Licence DR, Charnock-Jones DS.
416 Alternative splicing of vascular endothelial growth factor (VEGF)-R1
417 (FLT-1) pre-mRNA is important for the regulation of VEGF activity. *Mol*
418 *Endocrinol* 1999;**13**(4):537-45.
419 9. Toporsian M, Gros R, Kabir MG, et al. A role for endoglin in coupling eNOS
420 activity and regulating vascular tone revealed in hereditary hemorrhagic
421 telangiectasia. *Circ Res* 2005;**96**(6):684-92.
422 10. Li H, Gu B, Zhang Y, Lewis DF, Wang Y. Hypoxia-induced increase in soluble
423 Flt-1 production correlates with enhanced oxidative stress in trophoblast
424 cells from the human placenta. *Placenta* 2005;**26**(2-3):210-7.
425 11. De Marco CS, Caniggia I. Mechanisms of oxygen sensing in human trophoblast
426 cells. *Placenta* 2002;**23 Suppl A**:S58-68.
427 12. Zenclussen AC, Sollwedel A, Bertoja AZ, et al. Heme oxygenase as a
428 therapeutic target in immunological pregnancy complications. *Int*
429 *Immunopharmacol* 2005;**5**(1):41-51.

430 13. Zenclussen ML, Anegon I, Bertoja AZ, et al. Over-expression of heme
431 oxygenase-1 by adenoviral gene transfer improves pregnancy outcome in a
432 murine model of abortion. *J Reprod Immunol* 2006;**69**(1):35-52.
433 14. Ahmed A, Dunk C, Ahmad S, Khaliq A. Regulation of placental vascular
434 endothelial growth factor (VEGF) and placenta growth factor (PIGF) and
435 soluble Flt-1 by oxygen--a review. *Placenta* 2000;**21 Suppl A**:S16-24.
436 15. Cudmore M, Ahmad S, Al-Ani B, et al. Negative regulation of soluble Flt-1 and
437 soluble endoglin release by heme oxygenase-1. *Circulation*
438 2007;**115**(13):1789-97.
439 16. Farina A, Sekizawa A, DeSanctis P, et al. Gene expression in chorionic villous
440 samples at 11 weeks'gestation from women destined to develop
441 preeclampsia. *Prenat Diagn* 2008;**28**:956-961.
442 17. Okazaki S, Sekizawa A, Purwosunu Y, Iwasaki M, Farina A, Okai T.
443 Measurement of mRNA of trophoblast-specific genes in cellular and plasma
444 components of maternal blood. *J Med Genet* 2006;**43**(9):e47.
445 18. Poon LL, Leung TN, Lau TK, Lo YM. Presence of fetal RNA in maternal plasma.
446 *Clin Chem* 2000;**46**(11):1832-4.
447 19. Purwosunu Y, Sekizawa A, Farina A, et al. Evaluation of physiological
448 alterations of the placenta through analysis of cell-free messenger
449 ribonucleic acid concentrations of angiogenic factors. *Am J Obstet Gynecol*
450 2008;**198**(1):124 e1-7.
451 20. Purwosunu Y, Sekizawa A, Okazaki S, et al. Prediction of preeclampsia by
452 analysis of cell-free messenger RNA in maternal plasma. *Am J Obstet*
453 *Gynecol* 2009; **200**(4):386.e1-7..
454 21. Purwosunu Y, Sekizawa A, Farina A, et al. Cell-free mRNA concentrations of
455 CRH, PLAC1, and selectin-P are increased in the plasma of pregnant
456 women with preeclampsia. *Prenat Diagn* 2007;**27**(8):772-7.
457 22. Purwosunu Y, Sekizawa A, Koide K, et al. Cell-free mRNA concentrations of
458 plasminogen activator inhibitor-1 and tissue-type plasminogen activator are
459 increased in the plasma of pregnant women with preeclampsia. *Clin Chem*
460 2007;**53**(3):399-404.
461 23. ACOG practice bulletin. Diagnosis and management of preeclampsia and
462 eclampsia. Number 33, January 2002. American College of Obstetricians
463 and Gynecologists. *Int J Gynaecol Obstet* 2002;**77**(1):67-75.

464 24. Okazaki S, Sekizawa A, Purwosunu Y, Farina A, Wibowo N, Okai T.
465 Placenta-derived, cellular messenger RNA expression in the maternal blood
466 of preeclamptic women. *Obstet Gynecol* 2007;**110**(5):1130-6.
467 25. Collet D, ed. Modelling binary data. 2 ed. Boca Raton, Florida: CHAPMAN &
468 HALL/CRC, 2003.
469 26. Conde-Agudelo A, Villar J, Lindheimer M. World Health Organization
470 systematic review of screening tests for preeclampsia. *Obstet Gynecol*
471 2004;**104**(6):1367-91.
472 27. Espinoza J, Romero R, Nien JK, et al. Identification of patients at risk for early
473 onset and/or severe preeclampsia with the use of uterine artery Doppler
474 velocimetry and placental growth factor. *Am J Obstet Gynecol*
475 2007;**196**(4):326 e1-13.
476 28. Farag K, Hassan I, Ledger WL. Prediction of preeclampsia: can it be achieved?
477 *Obstet Gynecol Surv* 2004;**59**(6):464-82; quiz 485.
478 29. Sekizawa A, Purwosunu Y, Matsuoka R, et al. Recent advances in non-invasive
479 prenatal DNA diagnosis through analysis of maternal blood. *J Obstet*
480 *Gynaecol Res* 2007;**33**(6):747-64.
481
482

482 **Figure Legends**

483 **Figure 1a-g: Box and whiskers plot of the distribution of Flt-1, endoglin,**

484 **VEGF, PlGF, TGFβ1, HO-1, PLAC1, P-selectin and SOD mRNA levels in**

485 **controls and women with preeclampsia, stratified in accordance with the**

486 **severity of preeclampsia (mild, severe preeclampsia, and hemolysis,**

487 **elevated liver enzyme, low platelets (HELLP) syndrome)**

488 The medians are indicated by a line inside each box, the 75th and 25th percentiles by the

489 box limits; the upper and lower bars represent the 10th and 90th percentiles, respectively.

490 The Y-axes represent MoM values of each gene expression.

491 Asterisks (*) indicate all specimens above or below the 90th or 10th percentile specimens.

492 mild=mild preeclampsia, severe=severe preeclampsia, HELLP=HELLP syndrome

493

494 **Figure 2: ROC curve obtained by plotting the values of the**

495 **estimated odds for preeclampsia.** The multivariable model consisting

496 of ENG, FLT-1, PlGF and parity was used to generate the ROC curve.

497

497 **Table 1. Demographics characteristics.**

498 **Data are expresseed as median (Min-Max)**

	Controls (n=310)	Mild PE (n=26)	Severe PE (n=24)	HELLP (n=12)	p-value ∫*
% Nulliparous	59.8	65.4	87.5	91.7	0.008 §
Maternal age	28 (15-43)	32 (20-40)	27.5 (19-42)	24.5 (20-35)	0.065
GA at blood test (weeks)	17 (15-20)	18 (15-20)	16 (15-18)	17.5 (15-20)	0.479
BMI at blood test	22.94 (13.79-34.22)	24.93 (19.31-33.33)	21.93 (17.42-29.48)	21.71 (18.39-30.30)	0.150
SBP (mm Hg)at blood test	110 (90-130)	110 (90-125)	100 (90-120)	100 (90-130)	0.388
DBP (mm Hg) at blood test	70 (60-85)	70 (60-85)	70 (60-80)	70 (60-85)	0.625
Birth weight (g)	3195 (2600-4080)	2775 (1900-3920)	2500 (2100-3900)	2400 (1800-3130)	<0.001 §
Birth weight centiles	50 (14-94)	22 (1.5-92)	16 (1.5-55)	10 (1-90)	<0.001
GA at delivery (weeks)	38(37-41)	37(33-40)	37(34-40)	36 32-40)	<0.001 §

499 Data are expresseed as the median (Min-Max)

500 ∫ Chi square test

501 *Kruskall Wallis and Dunn test

502 Significant comparisons: § Control vs. severe PE and HELLP

503 Abbreviations: GA: gestational age; BMI: Body Mass Index; SBP: systolic

504 blood pressure; DBP: diastolic blood pressure

505

505 **Table 2. ROC curve for each marker**

mRNA	AUC	P-value	Lower 95% Bound	Upper 95% Bound	Sensitivity at 5% 1-specificity	Sensitivity at 10% 1-specificity	MoM Cut-off at 5% 1-specificity	MoM Cut-off at 10% 1-specificity
P-Selectin	0.665	0.039	0.588	0.742	18.2%	29.1%	1.61	1.45
PLAC1	0.631	0.044	0.545	0.717	20%	20%	1.31	1.26
Flt1	0.806	0.032	0.744	0.868	43.6%	52.7%	1.11	1.13
Endoglin	0.840	0.029	0.773	0.879	47.3%	50.9%	1.13	1.15
H0-1	0.588	0.042	0.502	0.675	8.1%	14.5%	0.84	0.87
PlGF	0.627	0.003	0.547	0.708	24.2%	25.8%	0.69	0.84

506 Sensitivity was shown at different cut-off values of 5%and 10% 1-specificity.
507 Abbreviation: AUC Area under curve, PlGF:placental growth factor;
508 HO-1:heme oxygenase-1; PLAC1: Placenta specific-1
509
510
511

511 **Table 3. Logistic regression output for plasma cellular RNA**
512 **levels**

Variable	Odds ratio	95% C.I.		p-value
Flt-1	2.760	1.910	3.988	<0.001
Endoglin	3.214	2.190	4.718	<0.001
PlGF	0.611	0.455	.821	0.001
Parity (primi or pluri vs. nulli)	2.822	1.316	6.052	0.008
Constant (ln)	-7.583			<0.001

513 The variables were expressed in MoM and categorized in 4 levels (<25th, 25th
514 - 50th, 50th-75th and >75th percentile)
515 Abbreviation:PlGF:placental growth factor1

Figure 1a

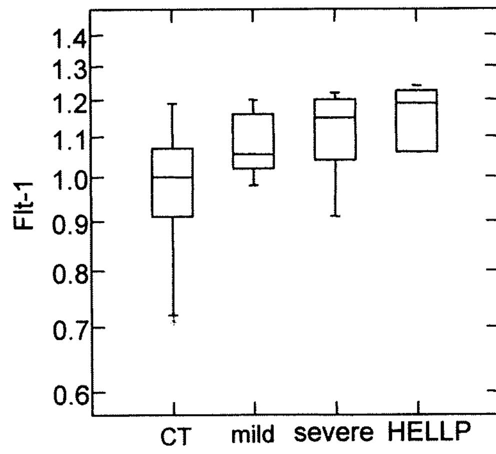

Figure 1b