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## **The Effects of Exercise on Urinary Erythropoietin Levels and Isoform Distribution**

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### **Introduction**

There have been a number of papers indicating that EPO levels in serum are affected by exercise but there are other papers showing no effect. Exercise of extended duration has been reported to elevate serum EPO levels with the greatest increase occurring some 30 hours after the event (Schwandt et al 1991). However other studies show no change in serum EPO after prolonged strenuous exercise (Weight et al 1992) and for short (1 hour) and long duration exercise (Klausen et al 1993). Later work also appears contradictory with one study showing that 3 minutes of supramaximal exercise causes elevated serum EPO 24 hours after (Roberts and Smith 1999) whilst another study showed no change in plasma EPO after acute exercise (Bodary et al 1999). It is likely that the claim will be made that exercise could possibly affect urinary EPO levels and potentially isoform distributions.

EPO produced under stress may have a different distribution of isoforms or the excretion behaviour of the isoforms may change. There is no evidence that such changes do occur but little has been published. This project was intended to measure urinary EPO levels and isoform distributions under varying forms of exercise to establish if such changes do occur.

### **Experimental**

Three groups were studied each representing varying degrees of exercise intensity: - the controlled group which exercised intensively to fatigue in less than 10 minutes, the City to Surf group who ran 14 km in 80 to 90 minutes, and the Trailwalker group who on two occasions ran 100 km cross country in approximately 27 hours.

The controlled group consisted of 6 females and 16 males with an age range of 18 to 31 years who exercised to perceived exhaustion under increasing load using a cycle ergometer or treadmill. The subjects were monitored by ECG and had blood lactate levels measured before and after exercise. Up to ten urine samples were collected from each subject.

The City to Surf Group consisted of 2 females and 3 males with ages of 31, 36, 41, 45 and 58. The "City to Surf" is an annual road race over 14 km with substantial hills. The winning time is approximately 45 minutes whilst the subjects took from 81 to 99 minutes. Up to ten urine samples were collected from each subject.

The Trailwalker Group consisted of 2 females and 2 males with ages of 24, 32, 59 and 59. The "Trailwalker" is an ultra-endurance charity teams event operated by Oxfam. It is a cross-country race over 100 km of difficult terrain. The participants are subjected to sleep and food deprivation as well as extreme fatigue. The winning time for a mixed team is approximately 17 hours. Our team took 27 hours. Several urine samples were collected from each subject over two runnings of the event.

The reason for choosing these three groups was that if there was a pronounced effect on

urinary EPO levels or isoform distributions then it would be anticipated that the effect would be -

- barely detectable for short duration (less than 10 minutes) exercise
- perhaps apparent for one to two hours exercise
- obvious after more than 24 hours of exertion.

#### Measurement of urinary EPO

To 1.2 mL of urine was added 24 uL of Complete protease inhibitor and 120 uL of 3.75M Tris-base at pH 7.4. The samples were centrifuged at 16,500 g for 10 minutes. 500 uL of each sample was transferred to a Microcon YM-30 device (MolecularWeight cutoff 30,000, Millipore, Australia) and centrifuged at 13,000 g for 10 minutes. The retentate was washed with 500 uL 50mM Tris-base and 10 uL Complete and centrifuged again. The volume of the retentate was adjusted to approximately 300 uL with 50mM Tris-base and transferred to an Immulite sample cup (Bio-Mediq DPC Pty Ltd, Australia). The EPO levels were then measured using an EPO kit (Immulite EPO chemiluminescent immunoassay, cat LKEP1, manufactured by Diagnostic Products Corp. Gwynned, UK) on a DPC Immulite instrument. Spiking trials have shown the method recovery to be 56% +/- 12%. The kit is provided for measurement of EPO in serum but is useful as a measure to get a idea of total EPO levels in urine to assess viability for the next step of isoform measurement.

#### Measurement of EPO isoforms

Each sample urine with a measured urinary EPO concentration of 4 mIU/mL or greater was subjected to isoelectric focussing using the Lasne method (Lasne et al 2002). To 20 mL of each urine was added 400 uL of Complete protease inhibitor and 2mL of 3.75M Tris-base at pH 7.4. This was concentrated by ultrafiltration to approximately 30uL using Centricon Plus 20 and YM-30 devices. The concentrated samples underwent isoelectric focussing on an ampholyte gel and then were transferred with two Western blotting steps. The membrane was visualised using Pierce SuperSignal West Femto Maximum Sensitivity Substrate (Pierce, USA). The signals were recorded with a Fuji LAS-1000 camera and quantitated with Fuji Image Guage v3.41 software. Wherever possible samples from the one subject were all run on the same gel to minimise inter assay variability. A typical gel is shown in Figure 1.

### Results and Discussion

The urinary EPO concentrations found for all the subjects prior to exercise are shown in Figure 2. The distribution is approximately normal. The variations in the urinary EPO concentrations over time for the controlled group were examined. Whilst for some subjects there are large variations in EPO concentrations there was no trend that can be related to exercise. A summary of the findings is shown in Figure 3. Similar results were found for the other exercise groups with no changes in EPO concentration that could be related to exercise. As this study was examining whether EPO isoforms vary in an individual it was necessary to first establish what variability there was in our % basic isoform measurements. Urine samples which had been spiked with varying levels of recombinant EPO from 0 to 15 IU/L were analysed between six and fifteen times on six gels run on different days. The results are shown in Table 1. As expected the variability was lowest with the high EPO levels.

Added EPO IU/L	Mean % basic isoforms	SD	CV
0	36.6	6.4	17.5
0.6	55.9	5.3	9.5
1.5	71.3	1.7	2.4
3	79.2	5.2	6.6
6	90.5	3.0	3.3
15	95.0	2.1	2.2

**Table 1 Variability of repeat measurements of % basic isoforms**

The results of the isoform analysis showed that some individuals have large variations in their % basic isoforms over time. These variations were much greater than any uncertainty associated with the measurement. However the variations did not seem to be related to the exercise but rather to natural variability. The results found for the female subjects in the group are shown in Figure 4 as an example. The short duration exercise in the controlled group would not be expected to have any significant effect on EPO production.

In the controlled group there were some 20 subjects with up to 10 urine samples from each which enables us to investigate whether the variability within one individual's % basic isoforms over time was greater or less than the variability found for the range of subjects at any one time. The results obtained are shown in Figure 5.

The range of variability observed in % basic isoforms for an individual is similar, although slightly smaller, to that found for the group but the spread of variabilities is somewhat greater for the group. Because of the large variations observed within all subjects it will be very difficult to detect any change in isoform distribution due to exercise unless the effect is large. The largest effect would be expected in the Trailwalker group who underwent the longest duration aerobic exercise. Figure 6 shows the % basic isoforms for each subject in Trailwalker 1 and Figure 7 shows the results for the same subjects in the second running of the event a year later. Figure 6 shows a rising trend in % basic isoforms for all four subjects with maxima occurring between 27 and 45 hours after the exercise began. Figure 7 shows a rising trend for three of the subjects but the fourth shows a decrease.

The results for the two runnings of the Trailwalker are summarised in Table 2. As the available literature indicated that serum EPO changes could be expected some 30 hours after aerobic exercise the samples taken more than 30 hours after the Trailwalker have been compared to those taken prior to the event. The comparison shows that there is a significant increase in % basic isoforms in the samples collected more than 30 hours after the event commenced for Trailwalker 1. This effect is less pronounced in Trailwalker 2. However the sample size is small and the exercise duration is very long being approximately 27 hours. No such changes were observed in the other two exercise groups with exercise durations of up to two hours.

As both urinary EPO concentrations and % basic isoforms had been measured for all subjects a comparison was made to see if there was any correlation between the two measurements. No correlation was found. Some representative results are shown in Figure 8.

Subject	Trailwalker 1		Trailwalker 2	
	Pre-event sample	First sample after 30 hours	Pre-event sample	First sample after 30 hours
S1	52	64	43	53
S2	33	57	28	57
S3	55	69	40	33
S4	45	51	50	74
Mean	46	60	40	54
Students t	0.017		0.09	

**Table 2 % Basic Isoform Results from Trailwalker 1 and 2**

### Conclusions

There is no evidence of changes in urinary EPO concentration or isoform distribution after short duration maximal exercise.

There was a general increase in % basic isoforms after 30 hours for those competing in the Trailwalker 1. The effect was less evident in Trailwalker 2. No sample would have been declared positive for recombinant EPO.

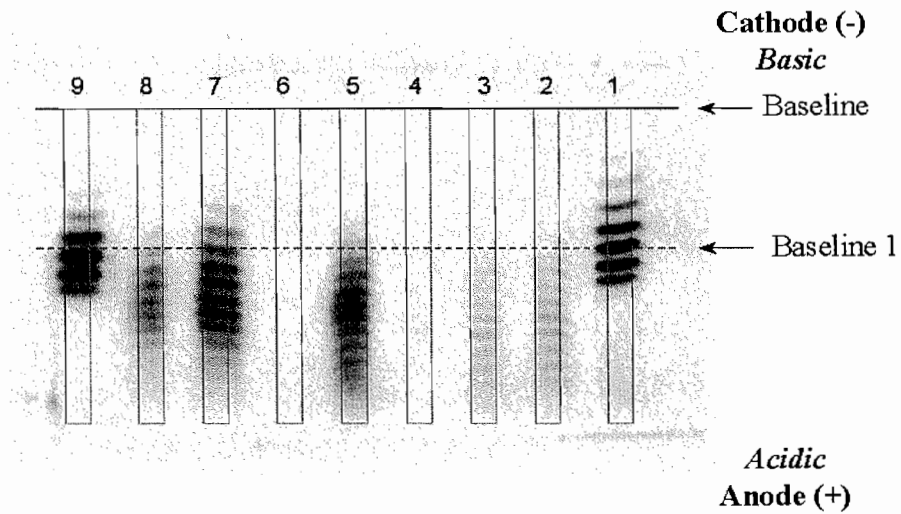
Further studies with more subjects are being undertaken to confirm whether the effect is related to exercise or merely represents normal variability.

### Acknowledgements

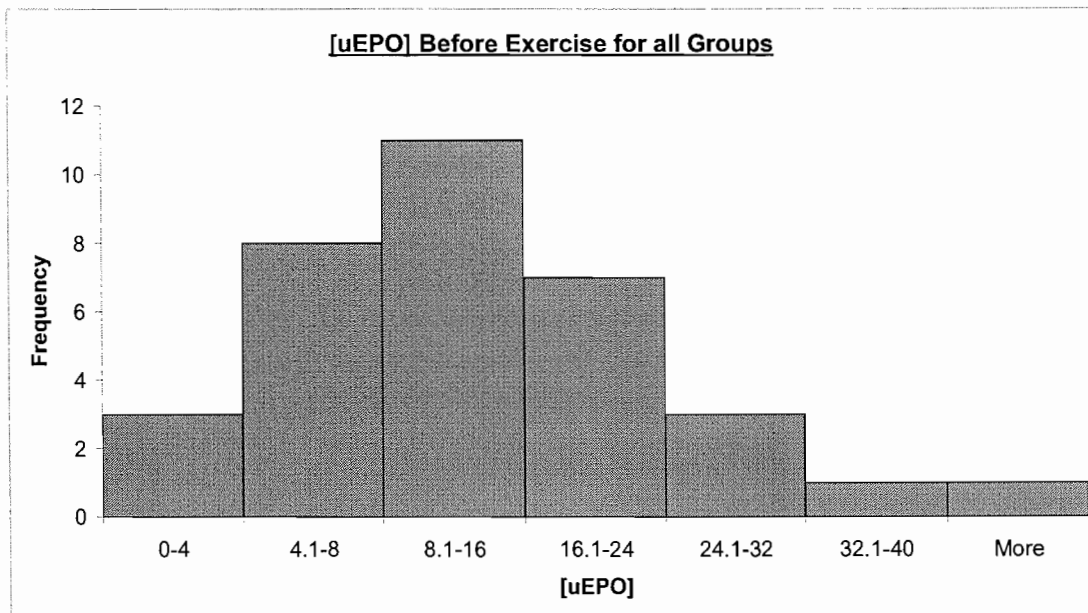
This project could not have proceeded without the generous support of the World Anti-Doping Agency. Our thanks go to all the subjects who gave of their time and effort in this study.

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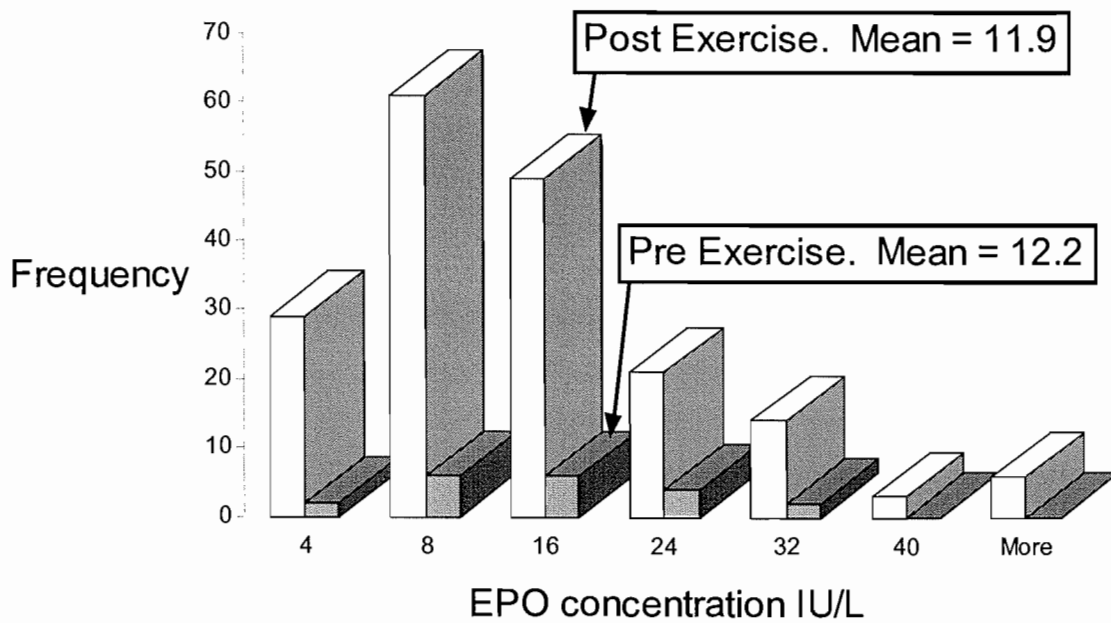
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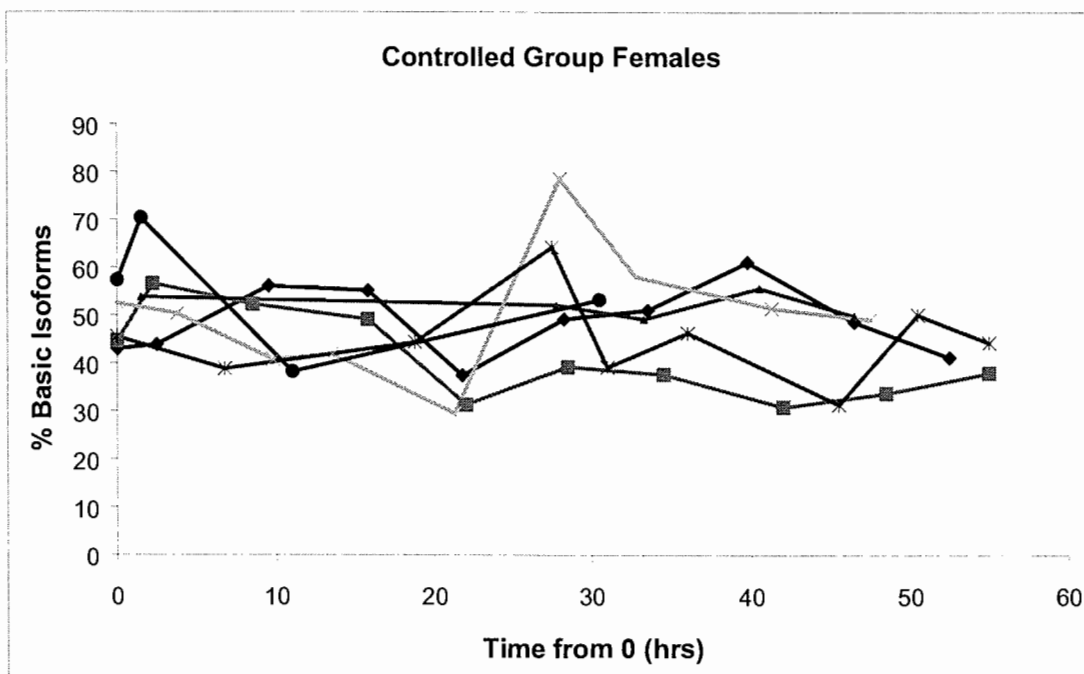
**Figure 1.** A typical gel with lanes 1 and 9 containing Erythropoietin Standard BRP-1 at 1500mIU/mL and lanes 2 to 8 containing concentrated urine samples.



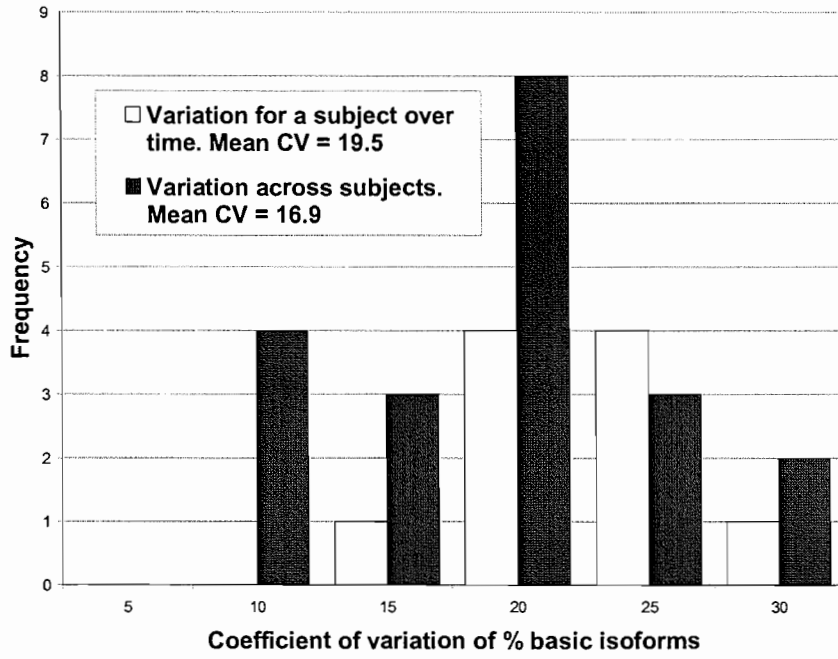
**Figure 2.** Frequency histogram of combined pre-exercise urinary EPO concentration results for all exercise groups.



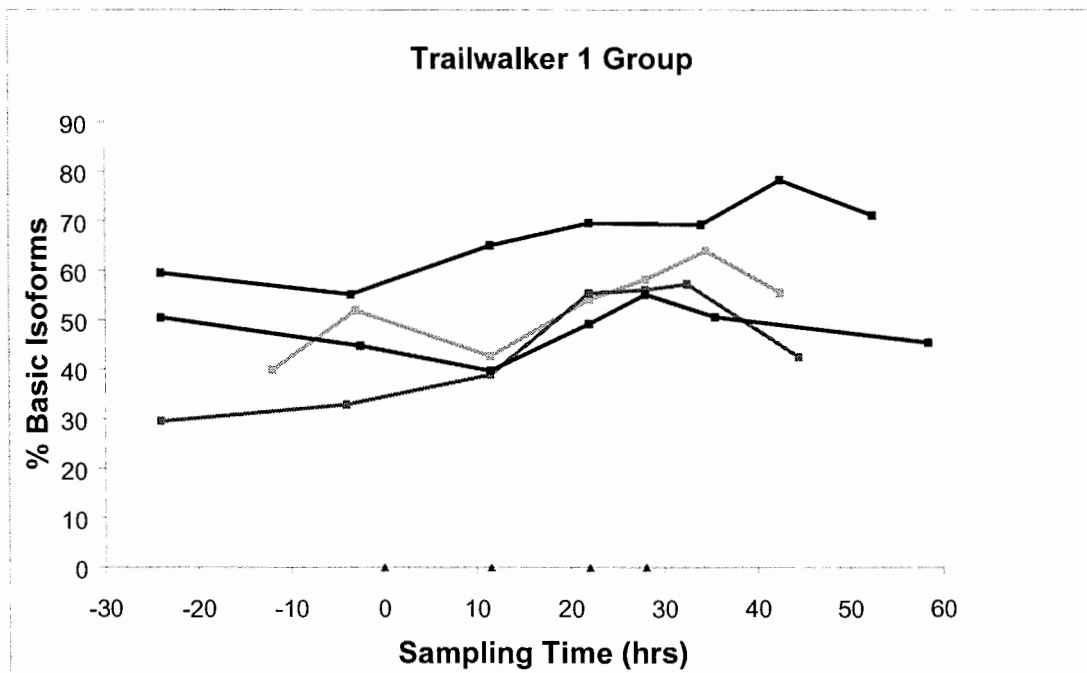
**Figure 3. Urinary EPO concentrations pre and post exercise for the controlled group.**



**Figure 4. % basic isoforms for the six females in the controlled group.**

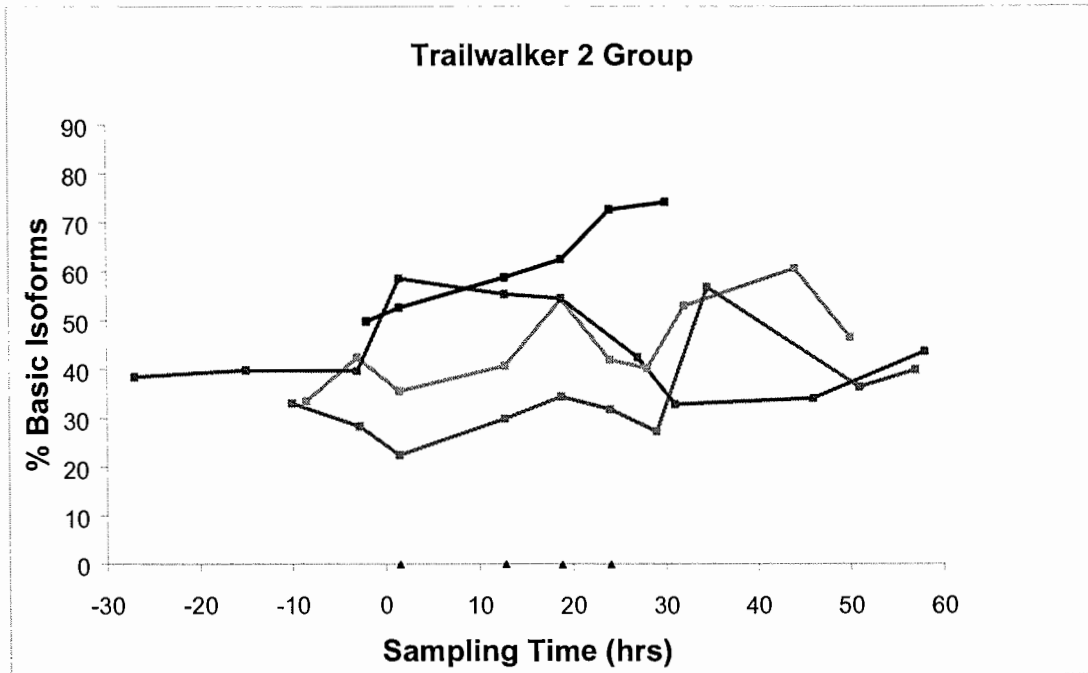


**Figure 5. Comparison of variability of % basic isoforms in controlled group.**

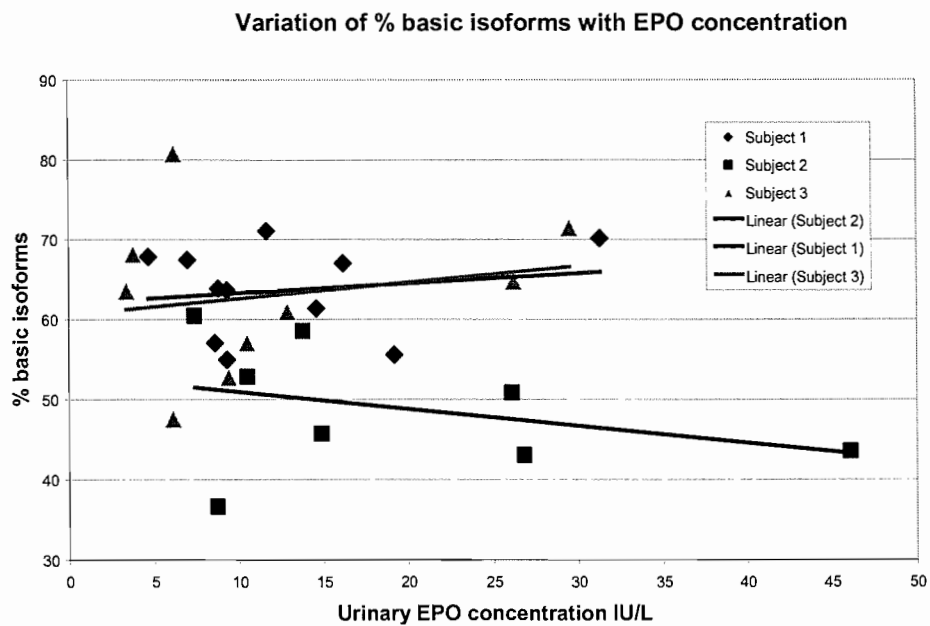


**Figure 6. Variation of % basic isoforms for the four Trailwalker 1 subjects.**





**Figure 7. Variation of % basic isoforms for the four Trailwalker 2 subjects.**



**Figure 8. Correlation between % basic isoforms and urinary EPO concentration.**