

# Normalization of Homocysteine in Dialysis Patients by Directed Repletion, with Apparent Reduction of Access Thrombosis

This report describes the first study in which all members of a group of dialysis patients had their homocysteine levels reduced into the normal range. The method utilized a lymphocyte transformation analysis to identify specific deficiencies in vitamins B6 and B12, folic acid minerals, glutathione, and antioxidant function at the functional, intracellular level. A supplementation program based on the results was designed for individual patients and consisted of N-acetylcysteine plus varying dosages of specific micronutrients that were determined to be functionally deficient. Within 12 weeks, the individualized treatment was found to reduce serum homocysteine levels into the normal range (mean = 12.8 pmol/L). After withdrawal of supplementation, the levels were found to remain low for at least an additional 25 weeks. It was observed that venous access thrombotic episodes were reduced by more than 50% during the 6 months following normalization of homocysteine concentrations. This appears to be the first reported instance of a direct physiological benefit associated with homocysteine reduction in dialysis patients.

Elevated blood homocysteine-which, to a mild or greater degree, is almost universal among dialysis patients-is now established to be a strong risk factor for cardiovascular mortality and morbidity in this population.<sup>1, 2</sup> As in the general population (in which hyperhomocysteinemia has a vastly lower prevalence), it is independent of classic risk factors such as increased cholesterol, LDL cholesterol levels, smoking, hypertension, obesity, and diabetes.'

In the non-end-stage renal disease (ESRD) population, a link between moderately elevated homocysteine concentrations and cardiovascular disease has implicated subclinical deficiencies of B vitamins as contributing factors. Selhub et al.<sup>4</sup> have demonstrated that approximately 67% of all cases of hyperhomocysteinemia are associated with one or more deficiencies of either vitamin B6, B12, Or folic acid. Elevations in homocysteine of 5 pmol/L are associated with a 60-80% increased risk of coronary artery disease.'Each 1 pmol/L increase in total homocysteine is associated with a 6% increase in the risk of developing cardiovascular disease.

Attempts to replicate the often successful'' normalization of homocysteine by treatment with B vitamins, which can be effected in the general population, have only met with limited success in dialysis patients. 11-16 The concept has thus gradually developed that dialysis patients are much more resistant to normalization of homocysteine than is the

general population.

The increased hyperhomocysteinemia seen among the end-stage renal disease population is also a thrombotic risk factor. Shemin et al. 17 reported that in a cohort of 84 hemodialysis patients with a fistula or prosthetic device as their primary hemodialysis access, 47 subjects (56%) had a least one-access thrombosis during an 18-month follow-up period. Proportional hazards modeling revealed that each 1 pmol/L increase in the total homocysteine level was associated with a 4% increase in the risk of access thrombosis.

This association persisted after adjusting for type of access, age, gender, diabetes, smoking, hypertension, dystipidemia, and the presence of previous vascular disease. Elevated homocysteine levels appear to confer a graded, independent risk for access thrombosis.

Whatever its cause, the incidence of thrombotic access events is much higher than even the elevated rate of cardiovascular mortality among dialysis patients. Beyond any consideration of the significant level of medical and financial resources that correction of the former pathology involves, its high incidence also offers an opportunity to observe any early positive effects of decreasing homocysteine levels in these patients.

**Table I. Vitamin repletion schedule for 20 of the 24 study patients (4 received a placebo). All the supplements were taken once daily for a 20-wk period; on dialysis days, supplements were taken post-dialysis.**

<b>Deficiency</b>	<b>Supplement</b>
<b>With Homosystiene &lt;16.5 <math>\mu\text{mol/L}</math></b>	
Deficiency of Vitamin B <sub>6</sub>	
50-55%*	10 mg daily
<50%	20 mg daily
Deficiency of Vitamin B <sub>12</sub>	
10-15%	10 $\mu\text{g}$ daily
<10%	1000 $\mu\text{g}$ daily
Deficiency of Folic Acid	
30-33%	1.0 mg daily
<30%	2.5 mg daily
Deficiency of Zinc	
125-160%	25 mg daily
>160%	50 mg daily, with 2mg copper for 3 mo, then reduce to 25 mg zinc daily
Deficiency of Spetrox	

<50%

60 mg vitamin C  
200 IU vitamin E  
(d-alpha succinyl tocopheryl)  
50ug selenium

Deficiency of Glutathione

500 mg N-acetylcysteine

### With Elevated Homocysteine >16.5 $\mu\text{mol/L}$

Deficiency of Folate

26-33%

2.5 folic acid  
1000 mg N-acetylcysteine

<26%

5.0 mg folic acid  
1000 mg N-acetylcysteine

Deficiency of Glutathione

<85%

1000 mg N-acetylcysteine

Elevation in Homocystiene Alone

>16.5  $\mu\text{mol/L}$

1000 mg N-acetylcysteine  
2.5 mg folic acid

*\*Functional intracellular assay results are expressed as a percentage of growth in a complete growth media. In interpreting reference ranges, in some assays, specific analytes are deleted from the growth media, and a failure of the lymphocytes to grow to predetermined levels is expressed in the results as "<X%" (e.g., Vitamin B<sub>12</sub>). For other assays, additional amounts of the analyte are added to the media, and a stimulation of growth is considered abnormal, and the results expressed as ">X%" (e.g., zinc).*

## Physiological Mechanisms

The metabolism of homocysteine is rather complex, involving a number of enzymes requiring a variety of organic and inorganic cofactors. The authors have previously<sup>18</sup> presented the concept that identifying the functional deficiencies of the specific cofactors involved in a particular homocysteine-metabolizing pathway has the potential likelihood of providing a guide to successful repletion. The "shotgun" approach-i.e., attempting repletion in the absence of relevant laboratory data-has, by comparison, a lower chance of success, as shown by the reported failures to actually normalize homocysteine levels in most patients.

What, then, constitutes relevant laboratory data? For this purpose, it is becoming clear that blood levels of the cofactors are of relatively little value. They often do not correlate with the results of functional analysis at the intracellular level at any given time, even if the blood compartment cofactor may eventually find its way into the cells. The only exception might be a situation in which a constant dose of a particular substance was given over a considerable period of time (weeks to months), leading ultimately to repletion at the intracellular level.

We have referred above to the concept that dialysis patients are relatively refractory to attempts at repletion. On a strictly empirical basis, this is certainly true. It may also be true in terms of physiological mechanisms, but here the conclusion may have to be modified in light of the fact that repletion studies up to the present have not adequately considered the specific repletion requirements of each individual patient. In other words, how much of the refractoriness is due to attempting repletion without knowing what is wrong with the patient?

The current investigation reports the effectiveness of normalizing homocysteine concentrations and decreasing the incidence of access thrombosis by first assessing functional vitamin, mineral, and antioxidant levels, and thereby formulating patient-specific supplementation programs.

# RATIONALE

The approach used in this study consists of

- 1) the measurement of homocysteine for each patient;
- 2) the determination of the intracellular vitamin/cofactor deficiencies related to homocysteine metabolism;
- 3) the initiation of a program to specifically correct each patient's functional cofactor deficit;
- 4) the redetermination of both homocysteine levels and the subcellular cofactors after the period of repletion; and
- 5) the observation of any effects on both the biochemical and physiological parameters affecting the patients.

**Table II. Functional intracellular and serum deficiencies pre-supplementation, measured post-dialysis (n = 24).**

Analyte	Mean (Nor*) (%)†	Mean (ABN^) (%)†	% ABN	No. ABN	Ref. Range†
<b>Functional Intracellular Deficiencies</b>					
Vitamin B <sub>6</sub>	67.4	47.2	20.8	5	>56%
Vitamin B <sub>12</sub>	23.1	13.0	50	12	>15%
Folate	44.3	28.2	33.3	8	>33%
Calcium	107.1	145.0	20.8	5	<130%
Magnesium	115.1	132.2	20.8	5	<124%
Zinc	121.6	128.6	41.7	10	<125%
Glutathione	114.8	70.61	50	12	>87%
<b>Antioxidant Function</b>					
<25%		23.5	8.3	2	>75%
26-75%		51.6	83.3	20	(optimal)
>75%		87.5	8.3	2	
<b>Serum Deficiencies</b>					
Homocysteine	13.1	24.6	91.7	22	<15.5 μmol/L
Vitamin B <sub>12</sub>	634.0	222.0	8.3	2	243-900 pg/ml
Folic Acid	18	4.7	4.2	1	5.3-14.4 ng/ml

\*Nor = Normal; ^ABN = abnormal; † = Functional intracellular assay results are expressed as a percentage of growth in a complete growth media. In interpreting reference ranges, in some assays, specific analytes are deleted from the growth media, and failure of the lymphocytes to grow to predetermined levels is expressed in the results as "<X%" (e.g., Vitamin B<sub>12</sub>) For other assays, additional amounts of the analyte are added to the media, and a stimulation of growth is considered abnormal, and the results expressed as ">X%" (e.g., zinc)

# MATERIALS AND METHODS

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Twenty-four subjects on peripheral renal hemodialysis were selected for participation in the study. Prior to providing supplements to these subjects, pre- and post-dialysis blood specimens were drawn for testing of functional metabolic deficiencies of vitamins B6 and B12, folic acid, calcium, magnesium, zinc, glutathione, and antioxidant function. In addition, blood specimens were obtained for the measurement of serum concentrations of homocysteine, vitamin B12, and folic acid.

All vitamins, antioxidants, and Nacetylcysteine were kindly provided gratis by Douglas, Apothocure, and College laboratories.

The functional assessment of intra-cellular analytes was performed using the lymphocyte growth assay as described by Shive et al.<sup>19,20</sup> In this procedure, blood is collected in a 10-ml cell preparation tube (CPT; manufactured by Becton Dickinson) containing sodium citrate and ficoll. The specimens are not processed in any manner, and are stored at room temperature. Upon overnight shipment to the laboratory, the specimens are centrifuged and the blood mononuclear cells (>90% lymphocytes) are isolated. The cells are washed to remove platelets and other contaminating cells, and the final lymphocyte preparation is diluted to a concentration of 150,000 cells/ml. Aliquots of the cell preparation are added to wells of a microtiter plate containing different modifications of CFBI 1000 media. The cells are stimulated to grow by the addition of phytohemagglutinin (PHA), and incubated at 37°C in a CO<sub>2</sub> incubator. Tritiated thymidine (<sup>3</sup>H) is added after 4 days, and incubation is continued for an additional 24 hours.

Cells from each well of the microtiter plate are harvested (via a Packard cell harvester), retaining cells containing <sup>3</sup>H-labeled DNA on filter paper. Radioactivity is determined by counting on a direct-read beta counter. All tests are performed in triplicate, and the results are expressed as a percentage of the cell growth in a complete serum-free media formulated for optimal growth with minimal concentrations of each media constituent.<sup>21-24</sup> Functional glutathione levels are measured by the inhibition of glutathione synthesis by butathionine sulfoxime (BSO).

The total antioxidant function (SpectroX™) is measured by the incubation and stimulation of the peripheral mononuclear cells in the complete, chemically defined, serum-free media, to which increasing concentrations of cumene hydroperoxide are added as a source of oxidative stress. The system measures the lymphocytes' capacity to resist oxidative damage.

Homocysteine was measured in serum by the method of Araki and Sako,<sup>25</sup> that utilizes high-pressure liquid chromatography. Homocysteine levels greater than 15.5 pmol/L were considered abnormal. Serum vitamin B<sub>6</sub> and folic acid were determined by radioimmunoassay.

Subjects were routinely dialyzed three times per week. Following the receipt of the pre- and post-dialysis testing results, specific vitamin formulations were prepared for each patient, as described in Table I. Nacetylcysteine was provided to all patients. This substance has the likelihood of aiding in the dialytic removal of homocysteine by reductively freeing the amino acid from any of its protein-bound disulfide forms.

All supplements were taken once daily for a 20-week period, and on those days when the subject was dialyzed, supplements were given post-dialysis by the nursing staff. Twenty patients received specific vitamin and antioxidant preparations, while 4 received a placebo. During the course of the study, each subject continued on his or her usual vitamin supplementation program, consisting of one standard renal-specific multi-vitamin taken daily.<sup>30, 31</sup>

**Table III. Functional intracellular and serum deficiencies pre-supplementation, measured post-dialysis (n = 10).**

Analyte	Mean (Nor*)	Mean (ABN^)	% ABN	No. ABN	Ref. Range†
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(%)†

(%)†

**Functional Intracellular Deficiencies**

Vitamin B <sub>6</sub>	72.8	49.0		1	>56%
Vitamin B <sub>12</sub>	27.8	12.5	80	2	>15%
Folatic Acid	44.7		0	0	>33%
Calcium	127.3	153.0	20	2	<130%
Magnesium	103.8	136.0	10	1	<124%
Zinc	96.5	127.0	10	1	<125%

**Antioxidant Function**

<25%	60.6 (n = 7)				(optimal)
26-75%	85.7 (n = 3)				
>75%					

**Serum Deficiencies**

Homocysteine	12.8	14.8	10	1	<15.5 μmol/L
Vitamin B <sub>12</sub>	731.0	234.0	10	1	243-900 pg/ml
Folic Acid	20.3		0	0	5.3-14.2 ng/ml

\*Nor = Normal; ^ABN = abnormal;

**RESULTS**

**Biochemical Changes**

The results of the pre-supplementation testing are shown in Table II. The mean homocysteine concentration was 24.6 pmol/L, with a range of 17.5-34.8 pmol/L. This is very similar to values reported for most dialysis patients.<sup>2, 26,27</sup>

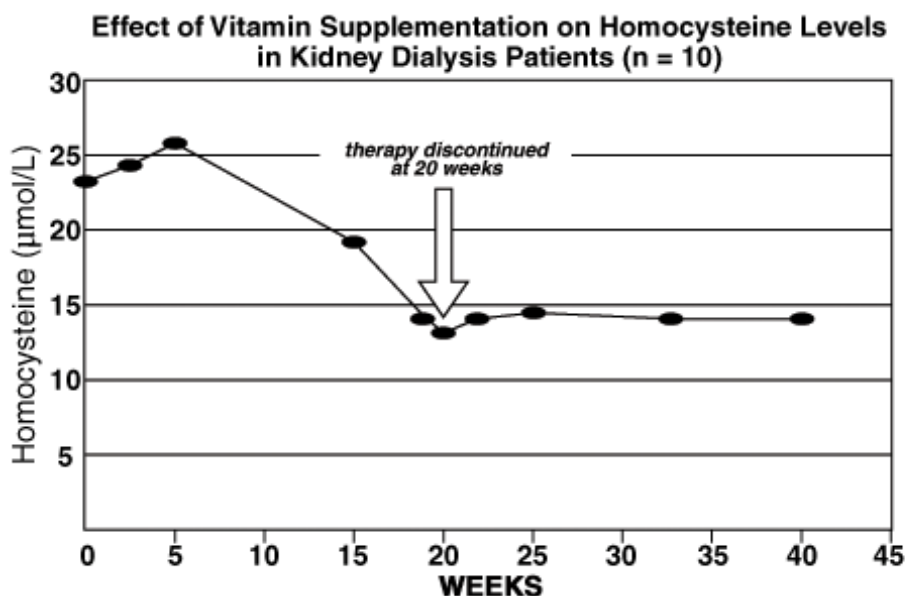
Functional intracellular deficiencies of vitamins B6, B12, and/or folic acid were found in 15 (62.5%) of the subjects. Specifically, deficiencies in vitamin B12 were noted in 50% of the subjects, deficiencies in vitamin B6 were noted in 20.8% of the subjects, and deficiencies in folic acid were noted in 33.3% of the subjects. Glutathione was deficient in 50% of the subjects, while antioxidant deficiency was found in about 90% of the subjects. This distribution of abnormalities approximates that previously found for a larger group of dialysis patients.<sup>28</sup>

Table II also shows the results of the serum testing that was performed on the pre-supplementation specimens following dialysis. Although each analyte was not tested in the serum, only 2 (8.3%) of the subjects were serum-deficient in vitamin B12, and 1 (4.2%) in folate. Serum testing did not reveal the cellular deficiencies identified by the functional assessment.

During the course of the 20-week supplementation program, 14 of the subjects discontinued their participation. Of those, 1 subject expired, 1 reported negative behavioral changes, and 12 were

considered non-compliant by the nursing staff. Those identified as non-compliant subjects frequently declined supplementation following their dialysis procedure, and no assurance could be obtained regarding compliance to the program outside the dialysis center. The single participant who expired was a placebo control, and 3 of the subjects considered non-compliant were also in the placebo group. Although not formally followed, it was observed through testing that the homocysteine levels of the placebo group did not non-nalize during the study period.

Table III summarizes the testing results following the 20-week supplementation period. Following the treatment period, the mean homocysteine concentration was 12.8 pmol/L, with a range of 9.8-14.6 pmol/L. Of the 13 subjects who were originally identified with functional deficiencies of vitamins B<sub>6</sub>, B<sub>12</sub>, and folate, 10 (76.9%) experienced corrected levels during the supplementation period. Further improvements were noted in the intra-cellular deficiencies previously determined for zinc and magnesium. One subject, whom was previously deficient in serum vitamin BB<sub>12</sub>, as well as being functionally deficient, remained deficient following post-supplementation testing. The post-supplementation homocysteine level for this patient was 14.6 pmol/L. Prior to implementing the supplementation program, the homocysteine level was 18.4 pmol/L.



### Duration of Homocysteine-Lowering Effect

The homocysteine levels, which were followed over the 20-week period, are shown in Figure 1. In the general population, treatment with N-acetylcysteine and B vitamins reduces homocysteine by approximately 45% in a 2-week period. After discontinuation of the supplementation program, the homocysteine returns to pretreatment levels within 2 weeks.

As shown in Figure 1, it required 12 weeks to initiate the change in homocysteine levels in our dialysis patient study group, with levels reaching the reported 12.8-pmol/L levels after 20 weeks. One of the most unexpected aspects of this study was that, unlike in the non-ESRD population, the homocysteine did not return to abnormal levels for at least 25 weeks after discontinuation of treatment.

## Access Thrombosis

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Following the conclusion of the study, a review of the incidence of access thrombosis was conducted on the remaining subjects and has continued through subsequent months. In a population of 24 dialysis patients followed for a 12-month period prior to the initiation of the repletion study—including 9 of the 10 patients who later completed the repletion study—an access thrombosis incident occurred on 15 occasions. This represents a rate of 0.62 incidents per patient per year.

Taking the entire grouping of patients who either completed or dropped out of the repletion study, the rate of thrombotic episodes during the succeeding 11 months fell to 0.41 incidents per patient per year. This is an improvement of 35.5% in this partially or fully repleted hybrid group. However, in the 7 months following the normalization of homocysteine concentrations after 20 weeks of repletion in the 10 fully treated subjects, there has been an incidence of only 1 thrombotic episode in this group. ***This represents an incidence of only 0.15 thrombotic episodes per patient per year, a decrease of 75.8%.*** During this same period of observation, all of the remaining patients, including the 10 who had been partially treated and the 4 who had not been repleted at all, showed an incidence of 0.33 thrombotic episodes per patient per year. Thus, the fully normalized group has improved by 53.3% over the hybrid group during this most recent observation period.

## DISCUSSION

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As demonstrated by the results of this study and numerous others referred to above, there is a prevalence of moderate to high hyperhomocysteinemia in the dialysis-dependent end-stage renal disease patient population.

Hemodialysis vascular access thrombosis is the most common cause of hospitalization among the maintenance dialysis population. The annual cost of this morbidity has been estimated at \$1 billion in the United States<sup>29</sup>. The underlying pathogenesis is unclear. Thromboses of arteriovenous fistulae or synthetic hemodialysis access grafts appear to result from stenotic lesions affecting arterial and venous blood flow. Homocysteine has been shown to be associated with the formation of these lesions.

In the general population, studies demonstrate homocysteine to be a risk factor for all forms of vascular disease, yet studies have not been published to indicate that the lowering of homocysteine levels through vitamin supplementation has any effect on the morbidity and mortality associated with vascular diseases. The unknown outcome of such repletion has reduced the attention of many in the medical community to treatment protocols designed to reduce homocysteine levels in the general population. Despite the lack of outcome studies, some research leaders recommend that if we have a safe, cost-effective treatment program, then our efforts should be directed toward reducing homocysteine levels in those patients at risk for vascular disease.<sup>32, 37</sup>

The results of the current study demonstrate that homocysteine levels can be normalized in the renal dialysis patient by identifying functional intracellular deficiencies of vitamins B6, B12, and folic acid, and then customizing individual supplement programs with appropriate B vitamins and N-acetylcysteine.

Homocysteine levels were reduced from a pre-supplementation mean of 24.6 pmol/L to a post-supplementation mean of 12.8 pmol/L, a reduction of 48.0%. All subjects had their homocysteine levels normalized (i.e., below or within the 11.0-15.0 pmol/L borderline range). The serum assessments did not reliably detect functional deficiencies, yet when patients were individually supplemented, homocysteine levels were reduced. In this regard, it is important to recall that many of these patients were functionally deficient in vitamin B6, B12, or folic acid, and that without further supplementation, normalization may not have occurred. Subjects will continue to be monitored to evaluate this process.

Although the design and objective of this study was to determine whether the high levels of homocysteine present in maintenance dialysis subjects could be normalized it was noted at the conclusion of the study that the incidence of thrombosis had been decreased in the successfully repleted patients. These data were obtained from a review of the quality assurance records at the dialysis center.

This present report represents a preliminary study of the results of repleting individual patients based on their individual functional deficiencies in cofactors related to homocysteine metabolism. As such, this study should be confirmed and extended by much larger studies. In addition, a host of critical additional questions could be answered by such studies: 1) How long will the lowered homocysteine level persist after cessation of repletion? 2) How long will corrections of intracellular deficits persist after repletion has been stopped? 3) Most importantly, can such repletion actually effect a reduction in cardiovascular mortality in dialysis patients, as well as a reduction of access thrombosis?

The currently reported decrease in access thrombotic incidents by directed repletion was a post-study observation and is, thus, almost semi-anecdotal. Nonetheless, the evidence is promising, and further studies in this area, including randomized and well-controlled clinical trials are required. The reduction in homocysteine levels almost certainly does not represent the limit of favorable results obtainable using this approach. A somewhat arbitrary choice of dosage of the repletion substances was made, which can presumably be optimized through later studies. Furthermore, neither the panel of intracellular tests nor the drug amiantarium employed are necessarily exhaustive. For example, the role, if any, of betaine and betaine-methionine transmethylase has not been evaluated in this preliminary study.

Finally, we would like to consider the question of why such a variety of intracellular cofactor deficiencies is seen in these patients, rather than only a specific few. The inference may be drawn that some general, rather than specific, process is at work that is affecting, at least potentially, a host of enzymatic processes.

It seems evident that homocysteine metabolism, despite its several pathways, has little excess capacity in the body. This putatively toxic amino acid is thus always on the threshold of increasing its concentration to undesirable levels. In almost all dialysis patients, this threshold is exceeded.

We have previously speculated that a possible mechanism might be the induction of conformational changes in the pertinent enzymes by a lowered intracellular pH, the ultimate cause of which is incomplete correction of the acidosis of renal failure. An even simpler possible etiology would be the effect of the elevated urea present in end-stage renal disease. Attention has been called<sup>38</sup> to the possibility that a micro-reversal of the Wohler urea synthesis first occurs, producing cyanic acid or its salts. This carbamylation reagent then irreversibly reacts with free amino groups on enzyme proteins, producing highly stable ureido derivatives with diminished enzymatic activity. Both of these chemical steps are non-enzymatic and are analogous to the formation of glycated proteins in high concentrations in diabetics.

The fact that the lowered homocysteine produced by the directed repletion persists for a remarkably long period after cessation of treatment might then be interpreted to mean that fresh enzymes have been induced,<sup>18</sup> which then may undergo cyanate damage only slowly. Since the kinetics of homocysteine lowering and raising by repletion/withdrawal seems so different in the dialysis patient than for the general population, the corollary under this hypothesis is that a special mechanism for homocysteine elevation in dialysis patients might be in play.

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