Title: TREATMENT OF VARICOSE VEINS

Abstract: Use of a modulator of an agent which regulates angiogenesis and/or a modulator of a nitric oxide synthase (NOS) in the manufacture of a medicament for use in the prevention or treatment of varicose veins, or of thread veins or venous flares in the absence of true the varicose veins.
TREATMENT OF VARICOSE VEINS

Field of the invention

This invention relates to the treatment of varicose veins.

Background of the Invention

The superficial veins of the lower limbs can become dilated and tortuous - varicose veins (VV's). The true prevalence of VVs can be difficult to determine, but it may be as high as 40% within the adult population, with an incidence increasing with age. Each year, up to half a million people with a venous disorder may consult their doctor, with women five-times more likely than men to do so. The cost overall to the United Kingdom National Health Service (NHS), to deal with VVs is considerable, running to hundreds of millions of pounds. As VVs are rarely life-threatening, however, they often command a low priority; one reason why VVs make up such a significant proportion of general and vascular surgical waiting lists.

Currently, four treatment options are available: do nothing; injection sclerotherapy; compression hosiery; and surgery.

1. Do nothing. The patient can be reassured that the condition is not threatening to life or limb. Masking creams may be used and the leg elevated and rested. Mild support hosiery may be used. Pregnant women and those planning to have children should be encouraged not to undergo surgical treatment. There is, however, a low risk of deep vein thrombosis (DVT).

2. Injection (or laser) sclerotherapy. This is sometimes suitable for venous flares, small reticular veins and minor VVs. The agent is deliberately inflammatory and is injected under direct vision by an experienced technician. Multiple treatments may be necessary and it can only ever be a temporary treatment as further VVs will develop. The procedure is uncomfortable and complications produce significant levels of litigation, because patient expectations are often high. Extravasation of the sclerosant causes skin to become stained brown, which may be permanent. In addition, tissue and skin necrosis (ulceration) may occur and DVT is a risk if the
agent tracks to deep veins. Typical agents used include sodium tetradecyl sulphate, ethanolamine olate and oxysclerol. Laser ablation again is uncomfortable, takes several treatments, is only temporary and is expensive (not offered by UK NHS).

3. Graduated compression hosiery. Graduated compression stockings come in three grades - 3, 2 and 1, giving progressively more compression at the ankle, reducing towards the knee, or thigh. They can be made as below-the-knee garments or as full length garments. The aim is to supplement the "calf muscle pump", which directs blood flow against gravity. For primary VVs, use is often confined to older persons, who are happy to wear them for prolonged periods each and every day.

Compression hosiery is uncomfortable in warm weather and is very unfashionable.

4. Surgery. Surgery is frequently undertaken on a day case basis, if the patient fulfills appropriate criteria, and usually if unilateral. A bilateral procedure, however, often requires an overnight stay. It is possible to operate on the long, or short saphenous system, or both. The surgeon marks the VVs prior to operation and for the long saphenous system, groin dissection is carried out under general anaesthetic to ligate and divide all LSV tributaries and the LSV is stripped to below the knee. Multiple stab avulsions of varices themselves along the thigh and calf may be carried out. A compression dressing is typically applied and the patient is often seen for a routine follow-up appointment. Complications of surgery include scaring, wound infection, wound haematoma, bruising and discomfort, recurrence and complications from anaesthetic.

Summary of the invention

We propose, for the first time, a molecular/cellular mechanism for the development of VVs. According to the invention, there is thus provided use of a modulator of an agent which regulates angiogenesis and/or a modulator of a nitric oxide synthase (NOS) in the manufacture of a medicament for use in the prevention or treatment of varicose veins, or of thread veins or venous flares in the absence of true varicose veins.

The invention also provides:
a method for preventing or treating varicose veins, or thread veins or venous flares in the absence of true varicose veins, in a host, which method comprises the step of administering to the host an effective amount of a modulator of an agent which regulates angiogenesis and/or a modulator of nitric oxide synthase (NOS); and

- an agent for the prevention or treatment of varicose veins, or of thread veins or venous flares in the absence of true varicose veins, comprising a modulator of an agent which regulates angiogenesis and/or a modulator of nitric oxide synthase (NOS).

**Brief description of the Figures**

Figure 1 shows levels of VEGF in the plasma from (A) control subjects and (B) patients with primary VVs. Each point represents the mean of triplicate experiments, each of duplicate wells/experiment. The bars represent the overall mean value for all results. Actual values and levels of statistical significance are presented in Table 1. "Arm" is the sample obtained from the arm before cuff application; "before cuff" is the sample obtained from the foot vein before application of the cuff; "after cuff" is the sample obtained from the foot vein after cuff application.

Figure 2 shows levels of NO in the plasma from (A) control subjects and (B) patients with primary VVs. Each point represents the mean of duplicate experiments, each of duplicate wells/experiment. The bars represent the overall mean value for all results. Actual values and levels of statistical significance are presented in Table 2. "Arm" is the sample obtained from the arm before cuff application; "before cuff" is the sample obtained from the foot vein before application of the cuff; "after cuff" is the sample obtained from the foot vein after cuff application.

**Detailed description of the invention**

Previously, simple mechanistic aetiologies have been proposed for the development of primary varicose veins (VV) and in particular, the concept of a
progressive, descending valvular incompetence (beginning at the sapheno-femoral
junction). Little attention has focused on the investigation of both the cellular and
molecular biological aspects of VVs, including an examination of the production,
release and receptor expression of those agents that mediate constriction and
relaxation of the vein wall.

We propose, for the first time, a molecular/cellular mechanism for the
development of VVs. Venous return from the lower limbs, back to the heart against
gravity, occurs primarily through changing intra-thoracic pressures and through the
action of the "calf muscle pump". Thus, regular use of the lower limb muscles helps
to ensure a satisfactory venous return, whereas if an individual stands still for
prolonged periods, venous return from the lower limbs may be slowed or impaired.
This may result in the following:

1. Relative venous stasis, with blood pooling within the veins.
2. A rise in intra-luminal venous pressure.
3. Lower limb swelling.

Of these three, venous stasis is, perhaps, the more important which will, in
turn, prompt the following:

1. A relative hypoxic state, due to the more prolonged presence of
deoxygenated blood.

2. A reduction in blood pH.
3. An accumulation of "waste products" (from tissue respiration).
4. An increase in the local concentration of reactive oxygen species (ROS).
5. A pooling of white blood cells; including macrophages, lymphocytes and
eosinphils.

6. A tendency towards the promotion of venous thrombosis.

The expected physiological response to a combination of any, of all, of these
factors should be to stimulate the vein wall to dilate and also, to become more
permeable - responses mediated directly by the production and release of VEGF and
NO. However, with this impetus to dilate, if an individual continues to stand
motionless, with the changes in intra-luminal pressure, stasis and blood chemistry not
balanced towards normal, the continuing physiological response to dilate and increase permeability might stimulate the further release of VEGF and NO. This process could continue as a potentially detrimental 'positive feedback loop' until such time as the stimulus for dilatation is ultimately relieved.

However, the vein wall itself will have a constrained capacity for dilatation due to both its' physical structure and secondarily, to those constraints placed upon it by the surrounding tissues. The situation whereby the stimulus to dilate is maintained but the vessel is effectively maximally dilated, could occur quite quickly. Here, if the stimulus is not relieved, then the production and release of VEGF and NO from the vein wall could continue, resulting in an ever increasing production and release of VEGF and NO. This positive "feed-back" might result in a dramatic potentiation of factors 1-6 mentioned above. In consequence;

1. NO is a ROS, whose continued production will promote the overall production of more damaging ROS locally.

2. NO is produced constantly at low levels by the constitutive form of the enzyme, NO synthase (NOS). However, the continued stimulus to produce NO will eventually exceed the capability of this particular enzyme form to produce NO. At this stage, there comes in to play an inducible form of NOS (iNOS) that can be activated, which results in the production of far greater levels of NO. However, iNOS is often associated with "pathology" (for example, it is the mechanism used most often by our immune system to combat invading foreign bodies). The very high levels of NO produced from iNOS often results in direct tissue damage, "oxidative stress", disturbance of cellular signalling and many other effects often associated with a pathological state.

3. NO combines readily with the superoxide anion ('O₂⁻'), a highly reactive oxygen species that is a by-product of normal cellular respiration. This leads to the formation of the even more damaging ROS, peroxynitrite (·ONOO). This degrades yet further to release ever more damaging ROS. Under normal conditions, the rate of combination of NO with 'O₂⁻' is limited by the dismutation (degradation) of 'O₂⁻' by anti-oxidant enzymes (superoxide dismutases - SODs) and also, the availability of
NO. However, with elevated NO production, the availability of NO to combine with \( \cdot \text{O}_2 \) becomes greatly increased and so, allows production of \( \cdot \text{ONOO} \). The overall effect is to produce increasing levels of ROS, of an increasing ability to directly damage tissue.

Thus, the continued stimulus to produce NO will exacerbate the 'positive feedback loop' problem further, as increased NO release will further increase the availability of ROS and the formation of \( \cdot \text{ONOO} \) due to both, enhanced NO availability and the ever increasing availability of \( \cdot \text{O}_2 \) from the accumulation of waste products from tissue respiration.

ROS can have the following effects on the vessel;

1. Stimulation of the breakdown of the extra-cellular matrix (ECM), resulting in the release of various cytokines (PDGF, VEGF, etc.) that may have in turn a damaging effect on the vessel wall.

2. Breakdown of the ECM will activate those enzymes responsible for breaking-down further the ECM and for vessel wall remodelling, such as collagenase, and matrix metallo-proteinases. The vessel wall may be stimulated to remodel and further cytokines will be released. The increased availability of these cytokines further exacerbates the problem.

3. Lowering the pH may once again stimulate a further breakdown of the integrity of the vessel wall and promote activation of vessel wall remodelling enzymes.

Here, overall, the vessel wall is trying its best to respond appropriately to the stimulus to dilate. Its' physical inability to dilate still further, because of wall structure and its' surrounds constrains this, however, but does not provide any simultaneous stimulus to prevent the production and release of those agents that try to further stimulate dilatation. So, in responding appropriately, the vessel wall reaches a stage whereby production of VEGF and NO continues, but these now result in an ever worsening "positive feedback loop". The down-stream effects of this excessive VEGF and NO production will be to damage the vessel wall and most probably, cause inappropriate vessel wall remodelling - varicosity. Indeed, the
pathology characteristic of VVs suggests inappropriate smooth muscle cell (SMC) proliferation and migration with damage resulting from the effects of ROS. Furthermore, although VVs demonstrate a reduced ability to constrict, or dilate, the vessel walls themselves maintain their ability to produce and release those agents mediating these responses. In the plasma of individuals with VVs, there are often higher than normal levels of precisely these agents. Continued exposure to such agents may, in effect, result in redundancy - whereby, the vessel actively down-regulates its' responses due to continued over exposure.

In summary, relative and/or prolonged venous stasis, or an increase in intraluminal pressure (perhaps, induced through prolonged standing) will tend to stimulate the vessel wall to dilate - a response mediated by the production and release of VEGF and NO. This stimulation will be maintained until such time as the venous circulation itself is enhanced by movement, activating the "calf muscle pump". If however, the stimulation is not relieved, the vein wall will continue to want to dilate until it has reached a state of maximal dilation. Once maximally dilated, if the stimulus for VEGF and NO production and release is still not relieved, a potentially damaging "positive feedback" cycle will be entered in to with further production and release of VEGF and NO. The continued stimulus for more NO will lead in turn, to the activation of iNOS and hence, very significantly elevated levels of NO. The overall effects on the vein wall will be; damage to proteins and interference with intra-cellular signalling, breakdown of the ECM, release of cytokines, activation of vessel wall remodelling enzymes and stimulation of SMC and endothelial cell (EC) migration and proliferation. With continued stimulus, the effects on the vessel wall will be to; promote a state of constant dilatation, cause aberrant SMC growth (resulting in a thickened intima), cause damage due to ROS, redundancy to normal physiological responses (receptor down-regulation) and the loss of controlled production and release of those agents that maintain vessel wall constriction and dilatation. Although these changes may be temporary and subtle, with repetition over time there will be a tendency to drift towards a pathological state. Humans, standing vertical on their "hind limbs" are the only species to suffer varicose veins.
Central to these processes are production and release of VEGF and NO. If oestrogen modulates production of VEGF and NO, the result may be to maintain higher background levels of either VEGF and NO (or their availability for release). Pre-menopausal women may be particularly at risk of developing VVs therefore, as a lesser stimulus to dilate would be required to achieve the higher levels of VEGF and NO necessary to initiate the "positive feedback" cycle and hence, initiate the pathology.

According to the invention there is thus provided use of a modulator of an agent which regulates angiogenesis and/or a modulator of a nitric oxide synthase (NOS) in the manufacture of a medicament for use in the prevention or treatment of varicose veins, or thread veins or venous flares in the absence of true varicose veins. The invention also provides a method of preventing or treating varicose veins, or thread veins or venous flares in the absence of true varicose veins, in a host, which method comprises the step of administering to the host an effective amount of a modulator of an agent which regulates angiogenesis and/or a modulator of a NOS. The host is a human. The invention also provides an agent for the prevention or treatment of varicose veins, or thread veins or venous flares in the absence of true varicose veins, comprising a modulator of an agent which regulates angiogenesis and/or a modulator of a NOS.

The condition of the varicose veins, or thread veins or venous flares in the absence of true varicose veins, of a patient suffering from any of those conditions could be improved by administration of a modulator of an agent which regulates angiogenesis and/or a modulator of a NOS. A therapeutically effective amount of a modulator of an agent which regulates angiogenesis and/or a modulator of a NOS may be given to a patient in need thereof. The invention also provides products containing a modulator of an agent which regulates angiogenesis and a modulator of a NOS for simultaneous, sequential, or separate use in the prevention or treatment of varicose veins, or thread veins or venous flares in the absence of true varicose veins.

Varicose veins, or thread veins or venous flares in the absence of true varicose veins, may be prevented or treated according to the invention. Typically,
the varicosity will be in the lower limb, for example the thigh or leg. In addition, varicosity of the haemorrhoidal veins (i.e. haemorrhoids) may be treated according to the invention. The term varicose veins is taken to indicate any dilated and/or tortuous veins. The term includes perhaps oesophageal varices, primary varicose veins, recurrent varicose veins, congenital arterio-venous malformations, congenital varicosities such as seen in the Klippel-Trenaunay syndrome, enlarged veins around a pregnant uterus, dilated veins from vena caval obstruction, following formation of renal access fistulae and any venous bed whose out-flow is obstructed by a mass. Also, the invention may be applied to the treatment of conditions associated with varicosity such as venous flares and thread veins.

Generally, the invention may allow prevention of varicosity formation. Furthermore, the invention may allow the degree of tortuosity and dilatation of varicose veins to be reduced. Also, any swelling, any aching or any discomfort may in turn be relieved. Ideally, the varicosity will substantially disappear, or will not progress, or will progress more slowly than it would without treatment according to the invention.

Typically, treatment according to the invention will be useful in the treatment of early varicose veins.

A human who is identified as at risk from developing varicose veins may be treated according to the invention to prevent varicose veins developing. Thus, the invention may be used as a prophylactic treatment.

In addition, treatment according to the invention may be combined with other types of therapy. Thus, treatment according to the invention may be carried out in conjunction with any other treatment for varicose veins, or thread veins or venous flares in the absence of true varicose veins, such as by sclerotherapy (for example by injection or laser), by surgery, or by compression hosiery. Treatment according to the invention may be carried out prior to, at the same time as, or following any other treatment for varicose veins, or thread veins or venous flares in the absence of true varicose veins.

The invention may be useful in the treatment of males or females, particularly
pregnant females. In addition, the invention may be useful in the treatment of older patients, for example a patient who is at least 40 years old, at least 45 years old, at least 50 years old, at least 55 years old, at least 65 years old, at least 70 years old, at least 75 years old or at least 80 years old.

A modulator of an agent which regulates angiogenesis, or a modulator of a NOS is an agent that modulates the normal function of an agent which regulates angiogenesis or of a NOS, such that the effects of the agent which regulates angiogenesis or of the NOS are reduced (i.e., the modulator may be an antagonist) or increased (i.e., the modulator may be an agonist). The modulator may exert its effect through direct modulation, for example a modulator which binds to an agent which regulates angiogenesis or a NOS itself, such that its activity is altered. Alternatively, the modulator may exert its effect through indirect modulation, for example a modulator which binds to a receptor for an agent which regulates angiogenesis, such that transduction of signalling is altered. Any pharmaceutically acceptable modulator of an agent which regulates angiogenesis or a NOS may be used in the present invention.

Preferred modulators for use in the present invention are modulators of positive regulators of angiogenesis, for example modulators of VEGF. Other preferred modulators are antagonists of an agent which regulates angiogenesis.

More preferred modulators are antagonists of positive regulators of angiogenesis, for example antagonists of VEGF.

As used herein, the term antagonist of a positive regulator of angiogenesis means any agent which inhibits the activity of a positive regulator of angiogenesis polypeptide, inhibits the expression of that regulator (i.e. the process of transcription and translation of, for example, VEGF) or inhibits signalling of the regulator via a receptor for the regulator. Typically, an antagonist specific for a particular positive regulator of angiogenesis is used.

As used herein, the term antagonist of a NOS means any agent which inhibits the activity of a NOS polypeptide or inhibits the expression of a NOS (i.e. the process of transcription and translation of a NOS). Typically, a NOS antagonist will
antagonise one particular NOS isoform more than it does any other NOS isoform. Preferably, a NOS antagonist will antagonise one particular NOS isoform, whilst having substantially no effect on other NOS isoforms. That is, ideally, the antagonist is a specific antagonist.

Any pharmaceutically acceptable modulator of an agent which regulates angiogenesis, for example a modulator, such as an antagonist, of a positive regulator of angiogenesis, can be used in the present invention. In particular, antagonists of vascular endothelial growth factor, for example vascular endothelial growth factor-A (VEGF-A) (Neufeld et al., 1994, Prog. Growth Factor Res. 5, 89-97) or VEGF-C (Ferrara, 1993, Trends Cardiovasc. Med. 3, 244-250), basic fibroblast growth factor (bFGF) (Folkman and Shing, 1992, J. Biol. Chem. 267, 10931-10934), hepatocyte growth factor (HGF) (Leung et al., 1989, Science 246, 1306-1309), angiopoietin-1, insulin-like growth factor-1 and -2 (Tischer et al., 1989, Biochem. Biophys. Res. Commun. 165, 1198-1206; Conn et al., 1990, Proc. Natl. Acad. Sc. USA 87, 2628-2633), epidermal growth factor (Maglione et al., 1991, Proc. Natl. Acad. Sc. USA 88, 9267-9271; DiSalvo et al., 1995, J. Biol. Chem. 270, 7717-7723), platelet-derived growth factor (Tischer et al., 1991, J. Biol. Chem. 266, 11947-11954; Park et al., 1993, Mol. Biol. Cell 4, 1317-1326) or sphingosine 1-phosphate (Maglione et al., 1993, Oncogene 8, 925-931) are useful in the invention. In addition, antagonists of the vasorelaxant prostacyclin may be useful in the invention. Typically antagonists of VEGF, preferably an antagonist of VEGF-A, are used in the invention.

Suitable VEGF antagonists include anti-VEGF antibodies and antigen-binding fragments thereof; receptor molecules which bind specifically to VEGF; agents which inhibit VEGF function, for example suramin and protein tyrosine kinase (PTK) inhibitors, for example lavendustin A (see for example, Waltenberger et al., 1996, J. Mol. Cell. Cardiol. 28, 1523-1529; Hu et al., 1995, Brit. J. Pharmacol. 114, 262-268. VEGF antagonists also include agents which inhibit binding of VEGF to VEGF receptors or extracellular domains thereof, for example platelet factor 4 (PF-4) (see for example, Gengrinovitch et al., 1995, J. Biol. Chem 270, 15059-15065). VEGF antagonists also include agents which inhibit VEGF receptor
signalling; and agents which inhibit VEGF activation, for example mithramycin (see for example, Ryuto et al., 1996, J. Biol. Chem. 271, 28220-28228). VEGF antagonists useful in the invention also include agents which are antagonists of signals that drive VEGF production, such as agents (for example drugs and other agents, including antibodies) which inhibit TGFβ or its ligands (see for example, Frank et al., 1995, J. Biol. Chem. 270, 12607-12613; Pertovaara et al., 1994, J. Biol. Chem. 269, 6271-6274). VEGF antagonists further include agents which are antagonists of signals that drive VEGF production and/or synthesis.

An anti-VEGF antibody suitable for use in the invention is typically capable of inhibiting the activity of VEGF in vivo. Preferably the antibody will specifically bind to VEGF. An antibody, or other compound, specifically binds to VEGF, i.e. does not bind substantially to other proteins. A variety of protocols for competitive binding or immunoradiometric assays to determine the specific binding capability of an antibody are well known in the art. Such immunoassays typically involve the formation of complexes between the specific protein and its antibody and the measurement of complex formation.

The antibody may be polyclonal or monoclonal. The terms polyclonal and monoclonal refer to the degree of homogeneity of an antibody preparation and are not intended to be limited to particular methods of production.

Anti-VEGF antibodies for use in the invention may be antibodies to full length human VEGF polypeptides or fragments thereof. For the purposes of this invention, the term "antibody", unless specified to the contrary, includes fragments of whole antibodies which maintain their binding activity for a polypeptide encoded by a polynucleotide of the invention, a polypeptide of the invention or a fragment thereof. Such fragments include Fv, F(ab') and F(ab')₂ fragments, as well as single chain antibodies. Furthermore, the antibodies and fragment thereof may be chimeric antibodies, CDR-grafted antibodies or humanised antibodies.

Anti-VEGF antibodies can be produced by any suitable method. Means for preparing and characterising antibodies are well known in the art, see for example Harlow and Lane (1988) "Antibodies: A Laboratory Manual", Cold Spring Harbor
Laboratory Press, Cold Spring Harbor, NY. For example, an antibody may be produced by raising antibody in a host animal against the whole polypeptide or a fragment thereof, for example an antigenic epitope thereof, herein after the "immunogen".

A method for producing a polyclonal antibody comprises immunising a suitable host animal, for example an experimental animal, with the immunogen and isolating immunoglobulins from the serum. The animal may therefore be inoculated with the immunogen, blood subsequently removed from the animal and the IgG fraction purified.

A method for producing a monoclonal antibody comprises immortalising cells which produce the desired antibody. Hybridoma cells may be produced by fusing spleen cells from an inoculated experimental animal with tumour cells (Kohler and Milstein (1975) Nature 256, 495-497).

An immortalized cell producing the desired antibody may be selected by a conventional procedure. The hybridomas may be grown in culture or injected intraperitoneally for formation of ascites fluid or into the blood stream of an allogenic host or immunocompromised host. Human antibody may be prepared by in vitro immunisation of human lymphocytes, followed by transformation of the lymphocytes with Epstein-Barr virus.

For the production of both monoclonal and polyclonal antibodies, the experimental animal is suitably a goat, rabbit, rat or mouse. If desired, the immunogen may be administered as a conjugate in which the immunogen is coupled, for example via a side chain of one of the amino acid residues, to a suitable carrier. The carrier molecule is typically a physiologically acceptable carrier. The antibody obtained may be isolated and, if desired, purified.

Humanized antibodies may be obtained by replacing components of a non-human antibody with human components, without substantially interfering with the ability of the antibody to bind antigen.

Preferably, anti-VEGF antibodies for use in the invention (and antigen binding fragments thereof) are characterised by high affinity binding to VEGF, for
example, high affinity binding to VEGF₁₂₁, VEGF₁₆₅, VEGF₁₈₅ or VEGF₂₀₆ and low toxicity (including HAMA and/or HACA response). An antibody where the individual components, such as the variable region, constant region and framework, individually and/or collectively possess low immunogenicity is particularly useful.

Examples of anti-VEGF antibodies are well known in the art, see for example Asano et al., 1995, Hybridoma 14, 475-480 and Kim et al., 1992, Growth Factors 7, 53-64.

VEGF receptor molecules for use in the present invention bind specifically to VEGF and possess low immunogenicity. Preferably, the VEGF receptor molecule is characterised by high affinity binding to VEGF. VEGF receptor molecules include VEGF receptors, such as tyrosine kinase receptors, KDR, Flk, for example Flk-1, and Flt, for example Flt-1 and Flt-4. See for example, Lee et al., 1996, Proc. Natl. Acad. Sc. USA 93, 1988-1992; deVries et al., 1992, Science 255, 989-991; Quinn et al., Proc. Natl. Acad. Sc. USA 90, 7533-7537; Shibuya et al., 1990, Oncogene 5, 519-524; and Terman et al., 1992, Biochem. Biophys. Res. Commun. 187, 1579-1586.

VEGF receptor molecules also include VEGF receptor multimeric molecules and VEGF immunoreceptor fusion molecules and derivatives and fragments thereof. VEGF receptor multimeric molecules can comprise all or a functional fragment of two or more VEGF receptors linked via one or more linkers. VEGF receptor multimeric molecules can further comprise a signal peptide of a secreted protein to direct expression of the multimeric molecule.

VEGF immunoreceptor fusion molecules can comprise at least one fragment of one or more immunoglobulin molecules and all or a functional fragment of one or more VEGF receptor(s). VEGF immunoreceptor fusion molecules can be assembled as monomers, or hetero- or homo-multimers. VEGF immunoreceptor fusion molecules can also be monovalent or multivalent. Examples of VEGF immunoreceptor fusion molecules are described by Aiello et al., Proc. Natl. Acad. Sci. USA 92, 10457-10461. See also Aiello et al., 1994 N. Engl. J. Med. 331, 1480-1487; Park et al., J. Biol. Chem. 269, 25646-25654.

A functional fragment or derivative of a VEGF receptor molecule refers to a
fragment of the VEGF receptor polypeptide or a fragment of the VEGF receptor polynucleotide sequence that encodes the VEGF polypeptide, that is of sufficient size and sequence to functionally resemble VEGF receptor molecules that can be used in the present invention (for example which bind specifically to VEGF and possess low immunogenicity). A functional equivalent of a VEGF receptor molecule also includes modified VEGF receptor molecules. For example, a functional equivalent of VEGF receptor molecule can contain a "SILENT" codon or one or more amino acid substitutions, deletions or additions. For example, a functional equivalent of VEGF receptor molecule can contain a substitution of one or more codon encoding the same or different hydrophobic amino acid for another coding encoding a hydrophobic amino acid. See Ausubel et al., Current Protocols in Molecular Biology, Greene Publishing Assoc. and Wiley-Interscience, New York (1989).

VEGF antagonists suitable for use in the invention may also be oligonucleotides and modified oligonucleotides or peptides, for example oligonucleotides as disclosed in EP-A-978561 or peptide as disclosed in WO-A-9929861.


Any suitable assay can be carried out to identify an antagonist of VEGF, or indeed an antagonist of any agent which regulates angiogenesis. For example, assays may be carried out to determine whether a test substance is capable of antagonising expression and/or translation of VEGF. Such assays may be carried out using cell based systems, or using cell free recombinant systems. Alternatively, a cell can be stimulated using VEGF in the presence of a test substance and the amount of NO release measured, for example by use of the Griess reagent. Reduced NO production as compared to a similar assay carried out in the absence of NO is indicative of the test substance being an antagonist of VEGF.
Any pharmaceutically acceptable antagonist of a NOS can be used in the present invention. Typically, an antagonist of a NOS enzyme is used. Competitive, non-competitive, reversible and irreversible antagonists are suitable. The antagonist may antagonise iNOS, eNOS and/or nNOS.

Suitable antagonists include L-arginine analogues, thioctirullines, indazoles, imidazoles, hydrazines, thioureas, thiazoles, biotins, phenyl-substituted thiopene amidines, pyridines, aminopyridines and aminoacid heterocyclic amides.

Examples of suitable L-arginine analogues include methyl-L-arginine, N⁰-nitro-L-arginine methyl ester (L-NAME), N⁰-monomethyl-L-arginine (L-NMMA), N⁰-amino-L-arginine (L-NAA), N⁰,N⁰-dimethyl-L-arginine (ADMA), N⁰,N⁰-dimethyl-L-arginine (SDMA), N⁰-ethyl-L-arginine (L-NEA), N⁰-methyl-L-homoarginine (L-NMHA), N⁰-nitro-L-arginine (L-NOARG), N⁰-iminoethyl-L-ornithine (L-NIO), N⁰-iminocaptyl-L-lysine (L-homo-NIO) and L-canavanine (L-CAN).

Examples of suitable thioctirullines include S-methyl-L-thiocittrulline (SMTC), L-thiocittrulline (L-TC) and L-S-ethyl-thiocittrulline (Et-TC).

Examples of suitable indazoles include indazole and 7-substituted indazoles such as 7-nitroindazole and 3-bromo-7-nitroindazole.

Examples of suitable hydrazines include aminoguanidine.

Examples of suitable imidazoles include phenyl substituted imidazoles such as 1-phenyl-imidazole.

Examples of suitable thioureas include S-methylisothiourea sulphate, δ-(S-methylisothioureido)-L-norvaline (L-MIN), S-ethylisothiourea (SETU) and S-isopropylisothiourea (SIPT).

Examples of suitable thiazoles include 2-amino-thiazole and 2-amino-4,5-dimethyl thiazole.

Examples of suitable biotins include 2-iminobiotin.

Examples of pyridines are disclosed in WO-A-0049015.

Examples of aminoacid heterocyclic amides are disclosed in WO-A-00/26195.

The above NOS antagonists are commercially available, or may be made by analogy with known methods.

The antagonists for use in the invention may be a pharmaceutically acceptable salt of one the above compounds. Suitable salts include salts with pharmaceutically acceptable acids, both inorganic acids such as hydrochloric, sulphuric, phosphoric, diphosphoric, hydrobromic or nitric acid and organic acids such as citric, fumaric, maleic, malic, ascorbic, succinic, tartaric, benzoic, acetic, methanesulphonic, ethanesulphonic, benzenesulphonic or p-toluenesulphonic acid. Salts may also be formed with pharmaceutically acceptable bases such as alkali metal (e.g. sodium or potassium) and alkali earth metal (e.g. calcium or magnesium) hydroxides and organic bases such as alkyl amines, aralkyl amines or heterocyclic amines.

Antagonists of NOS can be identified by:

(a) contacting a candidate agent with NOS and a substrate and co-factor therefor, under conditions under which NOS activity, in the absence of an inhibitor, would be expected to occur; and

(b) determining whether, or to what extent, NOS activity takes place.

A suitable such assay for identifying antagonists of NOS is a microtiter plate assay in which NOS activity is measured by determining the change in absorbance as NADPH is converted to NADP⁺. This assay comprises:

(a) adding a candidate compound, a known NOS antagonists (for example L-NMMA) and a buffer solution to separate microtiter wells;

(b) adding to each well NOS enzyme, cofactors therefor, L-arginine and buffer; and

(c) determining the change in absorbance in each well.

Typically, the buffer is a HEPES buffer capable of maintaining a pH of about 7, preferably about 7.4. The cofactors comprise oxyhemoglobin, NADPH and BHE. They may also comprise CaCl₂, MgCl₂, FMN, FAD and/or CaM.

The NOS may be a naturally occurring form of eNOS, iNOS, or nNOS or
may be a variant which retains NOS activity, for example variants produced by
mutagenesis techniques. NOS used in the assay is preferably of mammalian origin,
for example rodent (including rat and mouse) or primate (such as human).
Preferably, the NOS is of human origin.

The NOS may be obtained from mammal cellular extracts or produced
recombinantly from, for example, bacteria, yeast or higher eukaryotic cells including
mammalian cell lines and insect cell lines. Preferably, NOS used in the assay is
recombinant. More preferably, it is obtained by expression in S21 cells according to
the methodology in Charles et al., Methods in Molecular Biology (edited by M.A.
Titherage, Humana Press, Totowa), vol 100, pgs 51-60.

Step (c) of the assay may be carried out by reading the difference in
absorbance between 420 and 405 nm. Typically, this is done by a
spectrophotometer. Comparison of the well containing the candidate compound with
the control wells containing a known NOS inhibitor (100% inhibition) and no
inhibitor (0% inhibition) allows % inhibition achieved by the candidate compound to
be calculated.

A microtiter assay as set out above is described in detail in Dawson &
Knowles, Methods in Molecular Biology (edited by M.A. Titherage, Humana Press,

Suitable test substances for use in assays for identifying antagonists of VEGF
and/or a NOS include combinatorial libraries, defined chemical entities, peptides and
peptide mimetics, oligonucleotides and natural product libraries. The test substances
may be used in an initial screen of, for example, ten substances per reaction, and the
substances of batches which show inhibition tested individually. Furthermore,

antibody products (for example, monoclonal and polyclonal antibodies, single chain
antibodies, chimaeric antibodies and CDR-grafted antibodies) may be used.

Preferred antagonists are those which antagonise an agent which regulates
angiogenesis, for example VEGF, and/or a NOS by at least 10%, at least 20%, at
least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least
90%, at least 95% or at least 99% at a concentration of the inhibitor of 1 μg/ml.
µg/ml, 100 µg/ml, 500 µg/ml, 1 mg/ml, 10 mg/ml, 100 mg/ml as determined in an assay as described above. Preferably, the antagonist will achieve substantially total antagonism, that is preferably it will substantially completely inhibit activity in the assay.

The percentage antagonism represents the percentage decrease in "activity", however that is measured, in a comparison of assays carried out in the presence and absence of the test substance. Any combination of the above mentioned degrees of percentage inhibition and concentration of inhibitor may be used to define an antagonist for use in the invention, with greater antagonism at lower concentrations being preferred.

A modulator for use in the invention, for example an antagonist of VEGF and/or a modulator of a NOS is typically formulated for administration in the present invention with a pharmaceutically acceptable carrier or diluent. The pharmaceutical carrier or diluent will be selected depending on the particular modulator concerned. Depending upon the modulator, the carrier or diluent may be, for example, an isotonic saline solution. For example, solid, oral forms may contain, together with the active compound, diluents, e.g. lactose, dextrose, saccharose, cellulose, corn starch or potato starch; lubricants, e.g. silica, talc, stearic acid, magnesium or calcium stearate, and/or polyethylene glycols; binding agents; e.g. starches, arabic gums, gelatin, methylcellulose, carboxymethylcellulose or polyvinyl pyrrolidone; disaggregating agents, e.g. starch, alginic acid, alginates or sodium starch glycolate; effervescent mixtures; dyestuffs; sweeteners; wetting agents, such as lecithin, polysorbates, laurylsulphates; and, in general, non-toxic and pharmacologically inactive substances used in pharmaceutical formulations. Such pharmaceutical preparations may be manufactured in known manner, for example, by means of mixing, granulating, tabletting, sugar-coating, or film coating processes.

Liquid dispersions for oral administration may be syrups, emulsions and suspensions. The syrups may contain as carriers, for example, saccharose or saccharose with glycerine and/or mannitol and/or sorbitol.

Suspensions and emulsions may contain as carrier, for example a natural
gum, agar, sodium alginate, pectin, methylcellulose, carboxymethylcellulose, or polyvinyl alcohol. The suspensions or solutions for intramuscular injections may contain, together with the active compound, a pharmaceutically acceptable carrier, e.g. sterile water, olive oil, ethyl oleate, glycols, e.g. propylene glycol, and if desired, a suitable amount of lidocaine (lidocaine hydrochloride).

Solutions for intravenous or infusions may contain as carrier, for example, sterile water or preferably they may be in the form of sterile, aqueous, isotonic saline solutions.

A therapeutically effective amount of a modulator of VEGF and/or a modulator of a NOS is administered to a patient. The dose of modulator of VEGF and/or a modulator of a NOS may be determined according to various parameters, especially according to the particular modulator used; the age, weight and condition of the patient to be treated; the route of administration; and the required regimen. Again, a physician will be able to determine the required route of administration and dosage for any particular patient. A typical dose is from about 0.1 to 500 mg. Appropriate dosages may depend on a variety of factors, for example, body weight, according to the activity of the specific antagonist, the age, weight and conditions of the subject to be treated, the type and severity of the degeneration and the frequency and route of administration. Such a dose may be given, for example, once only, or more than once for example 2, 3, 4 or 5 times. The dose may be given, for example daily, every other day, weekly or monthly.
The following Example illustrates the invention:

EXAMPLE

Methods

This study was conducted following approval from the Joint UCL/UCLH Committees on the Ethics of Human Research. Patients attending the Vascular Unit, The Middlesex Hospital, London for the assessment for primary VVs and normal subjects from those within the Department of Surgery were asked to participate and then, consented.

All reagents, unless otherwise specified, were supplied by R&D Systems, UK. TWEEN, PBS, TBS, BSA, sucrose, carbonate and bicarbonate were purchased from Sigma, UK.

1. Induction of venous stasis

Control subjects (n=11; with "normal veins"), or patients with primary VVs (n=21) were rested supine. A foot vein was cannulated. Control subjects and patients, then continued to rest supine for a further 10 minutes. Subsequently, 5 ml of peripheral blood was collected (into EDTA; Vacutainer®) from the foot vein and 10 ml from an arm vein. Whilst remaining supine, a sphygmomanometer cuff was placed directly below the knee and inflated to 90-95 mmHg for 10 minutes to promote relative venous stasis. A further 5 ml of peripheral blood was then obtained from the foot vein (as before).

Within 15 minutes of collection, samples of peripheral blood were separated through Ficoll® (400 g from 25 minutes; brake off) and the plasma removed and transferred to CryoVials (Nune, UK) and stored at -20°C until analysed. The levels of plasma VEGF and NO were determined using commercially available kits (as detailed below).

2. Analysis of levels of plasma VEGF

Levels of plasma VEGF were determined using a commercially available
indirect ELISA assay kit (R&D systems, UK) and according to the manufacturer’s instructions.

Briefly, 96-well, flat-bottomed, Maxisorb® microtitre plates (Nunc, UK) were pre-coated overnight (at 4 °C) with capture antibody (0.4 μg/ml goat anti-human VEGF polyclonal; AF-293-NA) in carbonate/bicarbonate buffer (each 0.05M, pH 9.6). Antibody solution was discarded and the plates washed three times with wash buffer (0.05% TWEEN in PBS). Wells were blocked for 2 hours (room temperature) with blocking buffer (1% BSA, 5% sucrose in PBS) and then washed (x3, as described above). Plasma samples, or standards (7.8 - 4,000 pg/ml human recombinant VEGF165; 293-VE-010), diluted 1:1 in diluent (0.1% BSA, 0.05% TWEEN in PBS), were added and incubated for 2 hours (at room temperature). Samples were discarded and the plates washed (x3, as before). Detection antibody (0.2 μg/ml biotinylated goat anti-human VEGF polyclonal; BAF293) in diluent (as above) was added for 2 hours (room temperature) and then, plates washed (x3, as before). Extravidin®, conjugated to alkaline-phosphatase (Sigma, UK), diluted 1:50,000 in diluent (as above) was added for 30 minutes (at room temperature), plates washed (x3, as above) and substrate (1 p-nitrophenyl phosphate tablet [pNPP; Sigma Fast®] added to 1 tablet TBS in de-ionised water), made fresh and used within 15 minutes, added. Plates were incubated in a dark chamber for 60 minutes (at room temperature) before reading their optical density (OD) at 405 nm (Dynex microplate reader).

Standard curves (of 7.8 - 4,000 pg/ml recombinant human VEGF in doubling dilution) were performed for each experiment (duplicate wells/concentration). For analysis of samples, the mean of three experiments (each with duplicate wells/sample) was calculated and then used for further analysis.

3. Analysis of levels of plasma NO

Levels of plasma NO were determined by Griess Reagent (R&D Systems, UK) and according to the manufacturer’s instructions; the assay has been designed to measure levels of "total nitrites" (NO₂⁻); all reagents, unless otherwise specified, were
as provided by the manufacturer.

Briefly, samples were diluted 1:1 in reaction buffer (1x, diluted from 10x stock) and then to eliminate large proteins, ultrafiltered through a 10,000 MW cut-off filter (Millipore, UK). Samples, or standards (sodium nitrate, 3.125 - 100 μmol/L in doubling dilution), were added to the wells of a 96-well microtitre plate (as provided) and mixed 1:1 with reaction buffer (total volume, 100 μl). Next, 25 μl NADH (beta-Nicotinamide adenine dinucleotide) were added to each well, followed by 25 μl nitrate reductase. The plates were ‘tapped’ gently to mix the contents of the wells, covered with adhesive strip, and incubated at 37°C for 30 minutes. Following incubation, 50 μl of Greiss reagent I (sulfanilamide in 2N hydrochloric acid) and then 50μl of Griess reagent II (N-(1-naphthyl) ethylenediamine in 2N hydrochloric acid) were added to each well and the contents mixed by tapping. Next, plates were incubated for 10 minutes at room temperature and the OD recorded at 540 nm (Dynex microplate reader).

Standard curves were performed for each experiment and for analysis, the mean of two experiments, each with duplicate well/standard or sample, were calculated and used for further analysis.

Statistics

All results obtained experimentally were analysed first, by a one-sample Kolmogorov-Smirnov test for normality. Comparisons before and after cuff application, and between controls and experimental groups, were then made using the Student’s t test for paired, or unpaired results, as appropriate. All tests were two-tailed.

Results

The results obtained experimentally were of a normal distribution and have been presented as mean values ± s.e.m.

Twenty-one patients with primary VVs were enrolled into the study (6 males and 15 females; median age, 46 (range 21-78) years) and 11 control subjects (7 males
and 4 females; median age, 32 (range 22-55) years).

1. Release of plasma VEGF

The results are summarised in Table 1 and presented graphically in Figure 1.

In control subjects, the baseline levels of plasma VEGF obtained from the arm and those from the foot after rest, but before application of the cuff, were similar. However, following application of the below-knee cuff for 10 minutes, there was a clear trend for an increase in the levels of plasma VEGF; an overall increase in the mean level of plasma VEGF of 8.2% (P<0.1, Student’s paired t test; see Table 1 and Figure 1).

When examining the levels of plasma VEGF in patients with primary VVs, as with control subjects, baseline levels from the arm and the foot before application of the cuff, were similar. However, in contrast to control subjects, application of the below-knee cuff had little or no effect on the levels of plasma VEGF detected; a change in overall mean plasma VEGF of -1.5% (see Table 1 and Figure 1).

In all baseline samples (from the arm or foot, before or after cuff application), although not reaching statistical significance, the levels of VEGF detected in the plasma of patients with primary VVs were higher than those detected in the plasma from control subjects (see Table 1 and Figure 1); overall, a higher mean level of plasma VEGF of 28.1% for the "arm" and 20.5% for the "leg before application of the cuff". [After application of the cuff, in samples obtained from the foot veins, VVs had overall higher mean levels of plasma VEGF of 9.7%].

2. Release of plasma NO

The results are summarised in Table 2 and presented graphically in Figure 2.

In control subjects, the baseline levels of plasma NO from the arm, and foot after rest, but before cuff application, were similar. In contrast to VEGF, however, cuff application for 10 minutes had little or no effect on the levels of plasma NO detected.

In patients with primary VVs, the baseline levels of plasma NO from the arm,
and foot, after rest but before application of the cuff, were similar. Furthermore, as with control subjects, these remained mainly unchanged following application of the cuff.

However, in comparison to control subjects, in all samples from patients with primary VVs (from the arm, or foot, before or after cuff application), the levels of NO detected in the plasma were reduced (all \( P<0.05 \); for arm, or foot, before or after cuff application; all Student’s unpaired \( t \) test; see Table 2. and Figure 2.).

**Discussion**

Characteristically, VVs are dilated and tortuous, have aberrant smooth muscle cell (SMC) growth and distribution, and interruption of the layers of smooth muscle by elastin and collagen fibres suggesting collectively, an overall loss of the vein walls’ structural integrity (Thulesius *et al.*, 1988, Phlebology 3, 89-95; Travers *et al.*, 1992, Phlebology 7, 92-100; Rose and Ahmed, 1986, Cardiovasc. Surg. 27, 534-543; Obitsu *et al.*, 1990, Phlebology 5, 245-54). However, the contractility and reactivity of SMCs taken from VVs is similar to those from normal veins (Thulesius *et al.*, 1974, Angiology 35, 145-54). This suggests that the SMC component of the vein wall at least, should be able to function as normal. However, the vein wall must also be able to retain its’ ability to respond appropriately to the stimuli of changes in local physiology, for example, if there is lowered pH, or reduced oxygenation, the vein wall must primarily, be able to produce and release agents that mediate dilatation. Similarly, the vein must be able to balance these effects, by constricting. Does the appearance of VVs reflect a disruption in the balance between dilatation and constriction?

A number of recent studies using isolated "ring" sections of vein and organ bath pharmacology, have focussed on examining the change in response in VVs to agents that stimulate constriction and dilatation. Ring sections of VVs have a reduced ability to constrict (as judged by the maximal tension induced) in response to norepinephrine, angiotensin-II, KCL and endothelin-1 (ET-1) (Rizzi *et al.*, 1998, J. Vasc. Surg. 28, 855-61). Furthermore, the ability to constrict to these agents was
found to be related to the progression of varicosity (Rizzi et al., 1998, J. Vasc. Surg. 28, 855-61), an effect which, for ET-1, may be associated with a reduction in receptor expression as regulated at the transcription level (Barber et al., 1997, J. vasc. Surg 26, 61-9). Similarly, endothelium-dependent relaxation to calcium ionophore and endothelium-independent relaxation to either NO, or forskalin, was reduced (Lowell et al., 1992, J. Vasc. Surg. 16, 679-86 [Comment in J. Vasc. Surg 18, 138-9, 1993]).

In vivo, the most likely source of those agents mediating vascular reactivity is from the vein wall itself. However, to date, studies examining this function in the walls of VVs have been fairly limited.

Schuller-Petrovic and co-workers (1997) examined the plasma of normal individuals and patients with VVs (Schuller-Petrovic et al., 1997, Br. J. Pharmacol. 122, 772-8). With VVs, whilst levels of ET-1, cAMP and bradykinin were similar, levels of cGMP (the mechanism via which relaxation to NO is induced) were increased and angiotensin-II was reduced. Overall, the results suggested that ECs from VVs secreted fewer mediators of constriction, whilst increased cGMP/NO favoured dilatation. In separate studies, when examining the release of ET-1 in plasma following promoting of relative venous stasis (induced by a below-knee cuff), the results were contrasting (Mangiafico et al., 1997, Angiology 48, 769-74). Here, cuff application for 10 minutes induced a significant increase in levels of plasma ET-1 in both control subjects and patients with VVs, but the absolute increase in ET-1 was greater with VVs. Furthermore, and perhaps of greater importance, the baseline levels of plasma ET-1 before cuff application were significantly higher with VVs. Indeed, the reduced response to ET-1 in VVs associated with a reduction in ET-1 receptor expression may result from receptor "down-regulation" in response to an increased production of ET-1 (Mangiafico et al., 1997, Angiology 48, 769-74).

The main agents promoting dilatation are VEGF and NO (Thomas, 1996, J. Biol. Chem. 271, 603-6; Tomasian et al., 2000, Cardiovasc. Res. 47, 426-35). More recently, it has been appreciated that the interplay between these two molecules is central not only to dilatation and permeability, but also to the overall homeostatic
balance of the vessel wall (Servos et al., 1999, Cardiovasc. Res. 41, 509-10). Here, we have used the model of Magiafico and co-workers (1997, Angiology 48, 769-74) to examine the production and release of VEGF and NO in control subjects, compared to patients with VVs. The baseline levels of plasma VEGF (from samples taken from the arm, or foot, before cuff application) were higher in patients with VVs than control subjects (see Table 1.). Although this did not reach statistical significance, there is an approximate 20% elevation. Elevated levels of systemic VEGF have been reported previously with VVs, but only in association with skin changes (Shoab et al., 1998, J. Vasc. Surg. 28, 535-40). The increased availability of VEGF in the veins of the legs suggests that the problem with dilatation lies more with the inability of the vein to respond, rather than its' ability to produce VEGF. The elevated levels may be resultant upon a mechanism(s) similar to that reported for ET-1 (Barber et al., 1997, J. Vasc. Surg. 16, 679-86). The consequence here would be that if the vein wall had dilated maximally, it would be constrained from dilating any further due either to its' physical structure, or from its' opposition with the surrounding tissues. Alternatively, it may have a reduced ability to respond at all to VEGF stimulation. It would be interesting to record the density and distribution of VEGF receptors (KDR and flt-1) on, and within, the wall of VVs.

In contrast to VEGF, the baseline levels of NO in patients with VVs were significantly reduced compared to control subjects (all $P<0.05$; see Table 2.). It is unclear why these levels were reduced, as it might be expected from the elevated levels of VEGF that the levels of NO would be higher. Furthermore, this would be expected as NO is the primary agent mediating dilatation and VVs exist by definition in a state of constant dilatation. Reduced NO may have resulted from a reduction in the activity of (constitutive) NO synthase, damage to the endothelium (such that the stimulus for production is not responded to), or alternatively, to breakdown in the inter-play mechanism(s) with VEGF. Indeed, if VEGF functions to stimulate or enhance NO production (Servos et al., 1999, Cardiovasc. Res. 41, 509-10), loss of VEGF receptors, or receptor function within the VV wall, may help to explain the reduced levels seen.
After cuff application, to induce a relative venous stasis, control subjects demonstrated a clear trend (within 10 minutes) for increased levels of plasma VEGF \((P<0.1;\) see Table 1). With induced stasis, the stimulus must be for dilatation and additionally, to increase vein wall permeability. Therefore, the response would seem appropriate for the changes in venous blood chemistry that would be expected with stasis. However, there was little, or no, change in release of NO (see Table 2.). Why there was no associated increase in unclear, but it may be due to the short half-life of NO, methodological problems, or perhaps, more simply, to an inability to detect changes in the very small amounts of NO required to elicit a dilatory effect. In this respect, as with previous studies, it may have been more beneficial to examine changes in cGMP (Schuller-Petrovic et al., 1997, Br. J. Pharmacol. 122, 772-8).

With VVs patients, there was no change in either VEGF, or NO, after cuff application. In comparison to control subjects, the changes in the venous "environment" induced by cuff application were insufficient to stimulate the response to release VEGF (and perhaps, NO) and so, cause dilatation. This functional loss of release of VEGF, at least to the stimulus induced here, would tend to support the evidence gathered so far from *in vitro* studies, supporting the notion of a loss of vascular reactivity.

Overall, it might seem that with VVs there are elevated plasma levels of those agents that stimulate, or mediate, the responses of dilatation and constriction (Schuller-Petrovic et al., 1997, Br. J. Pharmacol. 122, 772-8; Barber et al., 1997, J. Vasc. Surg. 16, 679-86; Mangiafico et al., 1997, Angiology 48, 769-74) and yet, there is a relative loss of sensitivity to these agents. Consider, that if a vein is stimulated by "environmental" factors such as changes in local blood chemistry, the appropriate response to dilate, or constrict, should effect a balance towards normal, with the initiating stimulus having been withdrawn. If, however, the response is either insufficient or absent, then the tendency for the vein must be to continue to produce those agents required to produce the appropriate response (*i.e.* dilatation). For dilatation, this should be the production of VEGF or NO and for constriction, ET-1. Thus, with VVs, a loss of response to VEGF would explain the elevated levels
of this agent, as observed. Therefore, what could underlie the loss of response in the vein wall? A constant stimulus to dilate, as might be the case with prolonged standing, might result ultimately, in constant or over stimulation from elevated VEGF release in an active "down-regulation" of VEGF receptors. However, if the stimulus to dilate persists, there would be continued VEGF release and accordingly, NO production. If the interplay between VEGF and NO, central to the homeostatic balance of the vein wall becomes disturbed for prolonged periods, or repeatedly, this may lead eventually to the development of the pathology seen - varicosity.
### Tables

Table 1. Levels of plasma VEGF

<table>
<thead>
<tr>
<th></th>
<th>Control veins (ng/ml)</th>
<th>Varicose veins (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arm</td>
<td>4.81 ± 1.47</td>
<td>6.16 ± 2.13</td>
</tr>
<tr>
<td>Before cuff</td>
<td>5.03 ± 1.47</td>
<td>6.06 ± 2.06</td>
</tr>
<tr>
<td>After Cuff</td>
<td>5.44 ± 1.48*</td>
<td>5.97 ± 1.94</td>
</tr>
<tr>
<td>n=</td>
<td>9</td>
<td>12</td>
</tr>
</tbody>
</table>

Levels of plasma VEGF (ng/ml) in peripheral blood samples obtained from the arm and foot veins, before and after cuff application, in control subjects and patients with primary VVs. Values are presented as means ± s.e.m. "n" is the number of individuals whose complete results were available for analysis; each value represents the mean of triplicate experiments each of duplicate wells. "Before cuff" and "after cuff" are samples obtained from foot veins. *P<0.1, "before" versus "after" cuff application, Student's paired *t* test.
Table 2. Levels of plasma NO

<table>
<thead>
<tr>
<th></th>
<th>Control veins (pmol/L)</th>
<th>Varicose veins (pmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arm</td>
<td>46.9 ± 8.25</td>
<td>30.1 ± 2.36*</td>
</tr>
<tr>
<td>Before cuff</td>
<td>47.8 ± 7.89</td>
<td>32.4 ± 2.68*</td>
</tr>
<tr>
<td>After Cuff</td>
<td>48.0 ± 8.37</td>
<td>32.8 ± 2.61*</td>
</tr>
<tr>
<td>n=</td>
<td>11</td>
<td>20</td>
</tr>
</tbody>
</table>

Levels of plasma NO (pmol/L) in peripheral blood samples obtained from the arm and foot veins, before and after cuff application, in control subjects and patients with primary VVs. Values are presented as means ± s.e.m. "n" is the number of individuals whose complete results were available for analysis; each value represents the mean of duplicate experiments each of duplicate wells. "Before cuff" and "after cuff" are samples obtained from foot veins. *P<0.05, "normal" versus "varicose" for all samples (arm, leg before and after), Student’s unpaired t test.
1. Use of a modulator of an agent which regulates angiogenesis and/or a modulator of a nitric oxide synthase (NOS) in the manufacture of a medicament for use in the prevention or treatment of varicose veins, or of thread veins or venous flares in the absence of true varicose veins.

2. Use according to claim 1, wherein the modulator of an agent which regulates angiogenesis is a modulator of a positive regulator of angiogenesis.

3. Use according to claim 1 or 2, wherein the modulator of an agent which regulates angiogenesis is an antagonist of an agent which regulates angiogenesis.

4. Use according to claim 3, wherein the antagonist of an agent which regulates angiogenesis is an antagonist of vascular endothelial growth factor (VEGF).

5. Use according to any one of the preceding claims, wherein the NOS is a constitutive NOS or inducible NOS (iNOS).

6. Use according to claim 5, wherein the constitutive NOS is endothelial NOS (eNOS), or neuronal NOS (nNOS).

7. Use according to any one of the preceding claims, wherein the modulator of a NOS is an antagonist of a NOS.

8. Use according to any one of the preceding claims, wherein the medicament is used in the treatment of primary varicose veins.

9. Use according to any one of the preceding claims, wherein the medicament is used in combination with any other treatment for varicose veins, or for thread veins or venous flares in the absence of true varicose veins.

10. A method for preventing or treating varicose veins, or thread veins or venous flares in the absence of true varicose veins, in a host, which method comprises the step of administering to the host an effective amount of a modulator of an agent which regulates angiogenesis and/or a modulator of nitric oxide synthase (NOS).

11. An agent for the prevention or treatment of varicose veins, or of
thread veins or venous flares in the absence of true varicose veins, comprising a modulator of an agent which regulates angiogenesis and/or a modulator of nitric oxide synthase (NOS).