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Notice Of Entitlement

I, John David O'Connor, of 31 Market Street, Sydney, New South Wales, 2000, Australia, Patent Attorney for the Applicant/Nominated Person in respect of Application No. 11615/92 state the following:-

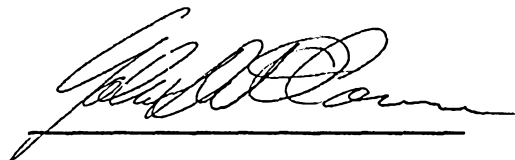
The Applicant/Nominated Person has entitlement from the actual inventors as follows:-

The Applicant/Nominated Person is the assignee of the actual inventors.

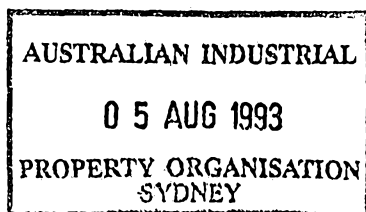
The Applicant/Nominated Person is the applicant of the basic application listed in the Declaration under Article 8 of the PCT.

The basic application listed in the Declaration under Article 8 of the PCT is the first application made in a Convention Country in respect of the invention.

Dated 30 July 1993



John David O'Connor





AU9211615

(12) PATENT ABRIDGMENT (11) Document No. AU-B-11615/92
(19) AUSTRALIAN PATENT OFFICE (10) Acceptance No. 658860

(54) Title
METHOD FOR DETECTION OF ANTI-RNA-ANTIBODIES

International Patent Classification(s)
(51)⁵ G01N 033/564

(21) Application No. : 11615/92 (22) Application Date : 16.01.92

(87) PCT Publication Number : WO92/13276

(30) Priority Data

(31) Number	(32) Date	(33) Country
91200121	22.01.91	EP EUROPEAN PATENT OFFICE (EPO)

(43) Publication Date : 27.08.92

(44) Publication Date of Accepted Application : 04.05.95

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(57) Claim

1. A method for monitoring an auto-immune disease activity by contacting U1-RNA with a testfluid of a patient with an auto-immune disease and detecting immune complexes formed between the U1-RNA and antibodies in the test fluid.

4. The method according to claim 1 or 2 characterized in that the U1-RNA comprises the B stem-loop domain of U1-RNA.

5. The method according to claim 1 or 2 characterized in that the U1-RNA comprises the E stem-loop domain of U1-RNA.

6. The method according to claim 1 or 2 characterized in that the U1-RNA

OPI DATE 27/08/92

APPLN. ID

11615 / 92

AOJP DATE 01/10/92

PCT NUMBER PCT/EP92/00106



INTERNA.....

TREATY (PCT)

(51) International Patent Classification ⁵ : G01N 33/564, 33/53	A1	(11) International Publication Number: WO 92/13276 (43) International Publication Date: 6 August 1992 (06.08.92)
(21) International Application Number: PCT/EP92/00106 (22) International Filing Date: 16 January 1992 (16.01.92) (30) Priority data: 91200121.1 22 January 1991 (22.01.91) EP (34) Countries for which the regional or international application was filed: AT et al. (71) Applicant (for all designated States except US): AKZO N.V. [NL/NL]; Velperweg 76, NL-6824 BM Arnhem (NL). (72) Inventors; and (75) Inventors/Applicants (for US only) : VAN VENROOIJ, Walther, Jacobus [NL/NL]; Eleonoraweg 16, NL-6523 MZ Nijmegen (NL). HOET, Rene, Antonius [NL/NL]; Weezenhof 3236, NL-6536 GK Nijmegen (NL).		(74) Agent: HERMANS, F., G., M.; Postbus 20, NL-5340 BH Oss (NL). (81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FI, FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, KR, LU (European patent), MC (European patent), NL (European patent), SE (European patent), US. Published <i>With international search report.</i> 658860
(54) Title: METHOD FOR DETECTION OF ANTI-RNA-ANTIBODIES (57) Abstract Method for the detection and quantitative determination of anti-RNA antibodies in a test fluid of patients with an auto-immune disease by contacting RNA with the test fluid by which a reaction occurs between the RNA and anti-RNA antibodies present in the test fluid after which a qualitative and/or quantitative determination of the antibodies reacting with the RNA provides information on the course of the disease.		

Method for detection of anti-RNA-antibodies

The invention relates to a method for monitoring an auto-immune disease activity by detection and quantitative determination of anti-RNA antibodies in a testfluid of patients with an auto-immune disease and a testkit for carrying out the method.

Since the discovery that sera of patients with systemic lupus erythematosus (SLE) contain antibodies to nuclear components, more refined methods have been developed to define the antigens involved in the reaction and to measure the concentration of antibody. Methods used to establish the nature of the antigen and antibody have included complement fixation, hemagglutination, precipitation of soluble immune complexes with ammonium sulfate, immuno-precipitation by double diffusion in agar and immuno-histology. The reaction has been quantitated by dilution of the sera for immuno-histology, by the extent of binding of DNA by the sera, and by solid-phase radio-immuno-assay. The use of antibodies coupled to enzymes has opened the possibility of using such conjugates to replace the radioactive label in immuno-assay systems.

Pesce et al (Clinical Chem. 20/3, 353-359, 1974) described a technique consisting of preparing an antigen on an insoluble support, layering the test-serum with anti-DNA, and using this serum as an antigen to react with an anti-human IgG labeled with enzyme-peroxidase. In principle, the amount of IgG bound to the insoluble support is proportional to the amount of antibody contained in the serum. Pesce et al demonstrated that a solid-phase non-competitive binding assay, with use of a second antibody conjugated to an enzyme as the quantitative or detector system, is a valid way to measure antibody to DNA in serum.

Patients with systemic auto-immune diseases produce a variety of antibodies directed against normal cellular components. Several well-characterized auto-antibody systems are directed against specific proteins or nucleic acid-protein complexes (Tan, E.M (1989) Adv. Immunol. 44, 93-151). Sera from patients with connective tissue diseases often contain antibodies against cellular components consisting of proteins associated with small RNA molecules of 80-200 nucleotides in length. Auto-antibodies against the nuclear type of these small RNA-protein complexes (snRNP) are mostly found in sera from patients with SLE or SLE overlap syndromes. Several specificities have already been described. There are antibodies that precipitate (U1)snRNP only, due to the fact that they recognise one or more of the (U1)snRNP specific proteins viz. 70K, A and C. Another specificity, anti-Sm, precipitates all the major nucleoplasmic snRNPS due to the fact that these antibodies recognise the proteins B'/B and D which are common to these snRNPS (v. Venrooij and Sillekens, Clin. Exp. Rheum., 1989).

The antibodies in the patients sera are in all these cases directed against proteins contained in the snRNP complexes. Although nucleic acids are generally poor immunogens, auto-antibodies directed against nucleic acids have been reported. The majority of studies of nucleic acid antibodies have concerned anti-DNA activities, although examples of anti-viral RNA auto-antibodies have been described.

Antibodies against the (U)RNA components of snRNP are thought to be rare (Tan, 1989). Wilusz and Keene described in 1986 (J. Biol. Chem, 261, 5467-5472) the presence of anti-(U1)RNA auto-antibodies in two anti-RNP sera. From this article no general conclusions regarding an association between anti-RNP and/or Sm activity and anti-(U1)RNA activity could be drawn. Deutscher and Keene described in 1988 (P.N.A.S., 85, 3299-3303) that the part of the (U1)RNA recognised by one of said sera was mapped and found to include the second stem-loop of the RNA.

We initiated a study in which a large number of anti-snRNP sera were tested for the presence of anti-(U1)RNA antibodies. Via screening with CIE (counter immuno-electrophoresis) and IB (immuno-blotting), 118 sera containing anti-snRNP activity were identified. Following RNP immuno-precipitation sixty sera were classified as anti-(U1)RNP and 18 sera as anti-Sm. Twenty-five sera contained anti-RNP as well as anti-Sm antibodies while 15 sera exhibited anti-(U1,U2)RNP activity.

These 118 sera were all tested for their ability to precipitate de-proteinized (U)RNA's. For this purpose total ^{32}P labeled Hela cell RNA, purified by extensive pronase and phenol/detergent treatment, but also in vitro synthesized (U)RNA's, was used as antigen in the precipitation assay. Immuno precipitation was carried out in IPP/500 mM NaCl, 10 mM Tris pH 7,5 and 0.05% Nonidet P40^(R). The overall results show that 45 out of the 118 sera (about 40%) contained anti-(U1)snRNA antibodies. These antibodies were not present in the anti-Sm sera. The auto-antibodies were always directed against (U1)snRNA and are most often present in patients with SLE overlap syndromes. Using immuno-precipitation under stringent conditions (500mM NaCl) with in vitro synthesized (U1)RNA or in vivo labeled total Hela cell RNA, it has been found that about 65% of the SLE overlap syndrome and about 30% of the SLE sera with anti-snRNP activity contained antibody against (U1)RNA. No auto-antibodies against other (U)RNAs were detected. Using in vitro synthesized stem-loops of (U1)RNA, it is possible to detect antibodies against all individual stem-loops of (U1)RNA. The major antigenic regions are however located in the so-called second (the B) and fourth (the E) stem-loop domain of (U1)RNA. It appears that the nucleotide sequence UUCG (nrs. 150-154) in the fourth E-stem loop is extremely essential as recognition site by patient specific antibodies.

The correlation between anti-(U1)RNA antibody level and auto-immune disease activity was most surprisingly.

According to a first embodiment of this invention, there is provided a method for monitoring an auto-immune disease activity by contacting U1-RNA with a testfluid of a patient with an auto-immune disease and detecting immune complexes formed between the U1-RNA and antibodies in the test fluid.

5 According to a second embodiment of this invention, there is provided a testkit when used for carrying out the method according to the first embodiment comprising a solid phase on which U1-RNA has been immobilized and means for detecting any immune complexes formed between the U1-RNA and antibodies in the test fluid.

According to the invention anti-RNA antibodies can be detected in a testfluid by
10 contacting RNA with said testfluid by which a reaction occurs between the RNA and anti-RNA antibodies present in the testfluid after which a determination of the antibodies reacting with the RNA may indicate and predict a change in the severity of disease.

Preferably above-mentioned determination of the antibodies reacting with the RNA will be a quantitative determination of the number of antibodies reacting with the U1-
15 RNA indicating a change in the severity of the disease.

The method according to the invention can be used to detect anti-(U1)RNA antibodies in serum from patients with the auto-immune disease SLE or SLE overlap syndrome.

In order to perform the method according to the invention the RNA used is
20 preferably labeled RNA, for instance labeled with a radio-active isotope or with biotin. However RNA attached to a solid phase like the innerwall of a micro-titerplate is also an option in order to perform the method.

The invention will be explained in more detail below.

EXAMPLE 1

25 Using a rapid and quantitative dot-blot binding assay as described below the correlation between disease activity and level of anti-(U1)RNA antibodies is demonstrated and outlined in figure 1. In said figure 1 is depicted that anti-(U1)RNA activity is predominantly found in patients with active disease. A typical dot-blot binding assay can be performed as follows. The labeled (U1)RNA transcript is made *in vitro* using T7
30 polymerase and (U1)cDNA. The RNA can be labeled in various ways (radioactive, with biotin etc.). The labeled RNA is incubated under certain conditions and in a buffer with diluted test fluid

from patients (serum, plasma etc.). The patient antibodies are able to bind the (U1)RNA and after equilibration has been reached (1 hr) the mixture is sucked off through a nitrocellulose sheet in a dot blot apparatus, using for example the Schleicher and Schull manifold with 96 wells. Under test conditions, naked RNA will not bind to the NC sheet, an RNA-antibody complex will. The NC sheet is washed, dried and detection of the labeled RNA bound on the NC via the antibody can be carried out, qualitatively and quantitatively. It is extremely important that the (U1)RNA offered to the test fluid is present in excess in order to obtain a linear correlation between concentration of antibody and amount of (U1)RNA bound. The method can also be performed using individual domains or epitopic regions of the (U1)RNA. This is important because in certain sera the severity of disease might correlate only with one class of antibody directed against only one epitopic region. In a three year prospective study disease activity indexes and levels of antibody activity against (U1)RNA were measured. Surprisingly, major disease exacerbations are associated with an increase of anti-(U1)RNA titer. This has now been found in more than 10 patients which were followed for 3 years or more. Strikingly, in the same patients no correlation between disease activity and antibody levels against the RNP-proteins (70K and A) could be found. By measuring the level of anti-(U1)RNA activity according to the invention it is now for the first time possible to monitor the disease activity and predict disease exacerbations which is extremely important for the assessment of the disease.

By using the method according to the invention a physician is able to predict to a certain extent that the patient will evolve into a worser condition. With this knowledge the physician can prescribe at the right moment certain medicaments that will bring the patient into a better condition or prevent a worsening of the disease (exacerbation).

EXAMPLE 2

According to the invention the method can also be performed using RNA attached to a solid phase. For such a purpose the wall of a small well in a micro titration plate to which said RNA is bound has often been used. The testfluid to be investigated is introduced in the small wells of the micro titration plate. The bound RNA is able to react with antibodies, specifically with anti-(U1)RNA antibodies, present in the serum from patients with SLE or MCTD syndrome as a result of which said anti-(U1)RNA antibodies are bound. In order to be able to detect whether an immuno-chemical reaction has taken place, subsequently one can use for instance marked antibodies which are directed against the auto-antibodies to be determined. Said marked antibodies will react with the RNA-auto antibody complex formed already on the solid phase. The marked antibodies consist of antibodies to which an enzyme is bound, preferably a peroxidase-enzyme. In the next step, a colourless solution (substrate + chromogen) is added. The enzyme is able to convert this solution into a coloured compound.

The intensity of the colour depends on the quantity of enzyme which is proportional to the quantity of bound auto-antibody. Said colour can be altered by adding a stop reagent.



The method thus described of determining an auto-antibody in the testfluid is a method of the sandwich type. Other immuno-chemical methods, such as an agglutination, inhibition or a competition reaction, can also be used. As suitable enzymes one can use, inter alia, alkaline phosphatase, urease and a previously mentioned peroxidase, preferably Horse Radish Peroxidase (HRP).

Instead of the above-mentioned wall of a small well in a micro-titration plate as solid phase one can use a tube or capillary, a membrane, filter, teststrip or the surface of a particle such as, for example, a latex particle, an erythrocyte, a dye sol, a metal sol or metal compound as sol particle.

Instead of above-mentioned enzymes as marker one can use as labelling substances, inter alia, a radioactive isotope, a fluorescent compound, a dye sol, metal sol or metal compound as sol particle.

The claims defining the invention are as follows:

1. A method for monitoring an auto-immune disease activity by contacting U1-RNA with a testfluid of a patient with an auto-immune disease and detecting immune complexes formed between the U1-RNA and antibodies in the test fluid.
- 5 2. The method according to claim 1 characterized in that the determination of antibodies reacting with the U1-RNA is a quantitative determination of the number of antibodies reacting with the U1-RNA, indicating a change in the severity of the disease.
3. The method according to claim 1 or 2 characterized in that the anti-RNA antibodies are anti-(U1)RNA reactive antibodies.
- 10 4. The method according to claim 1 or 2 characterized in that the U1-RNA comprises the B stem-loop domain of U1-RNA.
5. The method according to claim 1 or 2 characterized in that the U1-RNA comprises the E stem-loop domain of U1-RNA.
6. The method according to claim 1 or 2 characterized in that the U1-RNA
15 comprises a nucleotide sequence UUCG.
7. The method according to any one of the preceding claims characterized in that the U1-RNA is modified or labeled RNA.
8. The method according to any one of the preceding claims characterized in that the U1-RNA is attached to a solid phase.
- 20 9. The method according to any one of the preceding claims characterized in that the testfluid is serum, plasma or blood.
10. The method according to claim 9 characterized in that the testfluid is serum, plasma or blood from patients with a connective tissue disease such as SLE or SLE-overlap syndrome.
- 25 11. A testkit when used for carrying out the method according to any one of claims 1 to 10 comprising a solid phase on which U1-RNA has been immobilized and means for detecting any immune complexes formed between the U1-RNA and antibodies in the test fluid.
12. A method for monitoring an auto-immune disease activity by contacting
30 U1-RNA with a testfluid of a patient with an auto-immune disease and detecting immune complexes formed between the U1-RNA and antibodies in the test fluid, substantially as herein described with reference to Example 1 or 2.

Dated 15 February, 1995

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Anti-U1 RNA antibodies in serum Correlation with disease activity

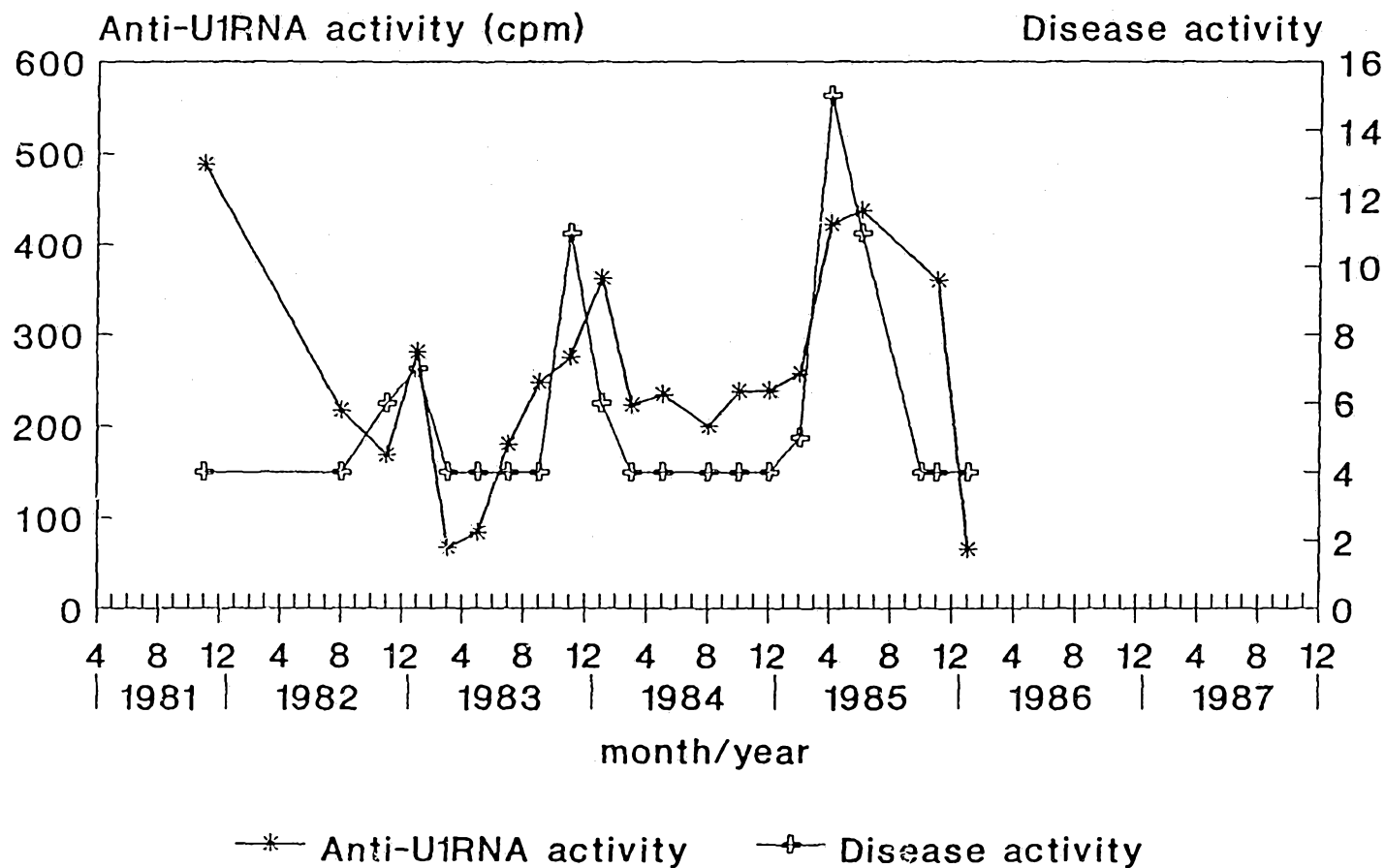


FIGURE 1

INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 92/00106

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC Int.Cl. 5 G01N33/564; G01N33/53		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
Int.Cl. 5	G01N	
Documentation Searched other than Minimum Documentation to the extent that such Documents are Included in the Fields Searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹		
Category ¹⁰	Citation of Document ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	REVUE DU RHUMATISME vol. 49, no. 5, April 1982, PARIS pages 385 - 391; GIOUD ET AL.: 'Etude des anticorps antihistones et anti-ARN bicaténaires dans les connectivites par une technique immunoenzymatique' see page 386 see page 388	1,2,7-9
X	IMMUNOLOGY LETTERS vol. 6, 1983, pages 33 - 38; ADAMASHVILI ET AL.: 'Antibodies to nucleic acids in systemic lupus erythematosus and rheumatoid arthritis patients and their relatives' see page 35	1,2,6,8, 9
Y	US,A,4 784 942 (HARLEY) 15 November 1988 see the whole document	1-4,6-10
¹⁰ Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
30 MARCH 1992	12.05.92	
International Searching Authority	Signature of Authorized Officer	
EUROPEAN PATENT OFFICE	CEDER O.	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES vol. 85, no. 10, May 1988, WASHINGTON, DC pages 3299 - 3303; DEUTSCHER ET AL.: 'A sequence-specific conformational epitope on U1 RNA is recognized by a unique autoantibody' cited in the application see the whole document ---	1-4,6-10
A	WO,A,9 010 229 (BIOSTAR MEDICAL PRODUCTS, INC.) 7 September 1990 see claims 14,15 ---	1,2,7-10
A	THE JOURNAL OF BIOLOGICAL CHEMISTRY vol. 261, no. 12, 25 April 1986, BALTIMORE, MD pages 5467 - 5472; WILUSZ ET AL.: 'Autoantibodies specific for U1 RNA and initiator methionine tRNA' cited in the application see the whole document ---	1-3,6,8, 9
A	CLINICAL CHEMISTRY vol. 20, no. 3, March 1974, WINSTON-SALEM, NC pages 353 - 359; PESCE ET AL.: 'Use of enzyme-linked antibodies to measure serum anti-DNA antibody in systemic lupus erythematosus' cited in the application see abstract ---	1,2,7-9

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.**

EP 9200106
SA 55231

This annex lists the patent family members relating to the patent documents cited in the abovementioned international search report.
The members are as contained in the European Patent Office EDP file on
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A-4784942	15-11-88	None	
WO-A-9010229	07-09-90	AU-A- 5186890	26-09-90
		EP-A- 0460102	11-12-91