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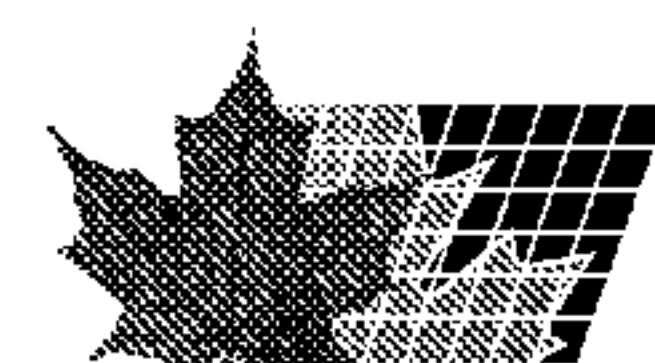
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(54) **Titre : ANTIGENES DE CHLAMYDIA TRACHOMATIS UTILISES A DES FINS DE VACCINATION ET DE DIAGNOSTIC**

(54) **Title: CHLAMYDIA TRACHOMATIS ANTIGENS FOR VACCINE AND DIAGNOSTIC USE**



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ABSTRACT .

The present invention is related to antigens from *Chlamydia trachomatis* which are recognized by specific antibodies from individuals infected with Chlamydia or which can induce T cells from the same individuals to secrete gamma-interferon. The T cell reactive antigens are present in a whole-cell lysate and have apparent molecular weights of 5-12, 16-20, 25-35, and 58-74 kDa as determined by SDS-PAGE. The antigens of the invention are useful in vaccines but also as diagnostic compositions.

Chlamydia Trachomatis antigens for vaccine and diagnostic use.

This is a division of Canadian patent application No. 2,585,011
5 filed on October 11, 2005.

Field of invention

The present invention discloses the use of immunogenic polypeptides and immunogenic
compositions based on polypeptides and nucleic acid derived from *C. trachomatis* as
10 vaccine and diagnostic agents.

General Background

Chlamydial species cause a wide range of diseases in both animals and humans. Of
particular concern is *C. trachomatis*, an obligatory intracellular bacterium, which infects
15 and multiplies in epithelial cells. It is the most frequent cause of sexually transmitted
disease (STD) in developed countries and it is the most common cause of ocular disease in
developing countries (Schachter, Moncada et al. 1988). There is an estimated 92 million
individuals who carry the infection globally (WHO, 1999).

20 The duration of untreated Chlamydia STD is prolonged, and complete clearance is often
not reached within the first 12 months. The protective immunity induced during the
infection is thought to be serovariant-specific and short-lived, thus allowing frequent re-
infections (Katz, Batteiger et al. 1987). These circumstances, the prolonged course of
infection and the possible re-infections may lead to the development of serious sequelae,
25 including pelvic inflammatory disease, infertility and ectopic pregnancies (Brunham
1999).

The infection is effectively controlled by antibiotic therapy; however the high prevalence
of asymptomatic cases suggests that sustainable Chlamydia control can only be envisaged
30 if an effective Chlamydia vaccine is developed. While much effort has been devoted to a
vaccine against Chlamydia infections over the last few decades, so far no vaccine has been
developed.

This makes the development of a vaccine against Chlamydia an urgent matter. Many
35 attempts to define protective chlamydial substances have been made, however, the
demonstration of a specific long-term protective immune response has not yet been

achieved. Over the last several decades much effort has been devoted into developing a vaccine against Chlamydia infections however, so far no vaccine has been developed. Some of the first efforts were focused on controlling trachoma, and whole viable or inactivated organisms were used as the antigen to immunize humans and monkeys (Wang, 5 Grayston et al. 1967; Grayston and Wang 1978). Children vaccinated with an inactivated whole-cell vaccine initially resulted in protection but the protection was serovar specific and short-lived (Grayston and Wang 1978). Furthermore, reinfection of partially protected individuals resulted in clinical disease that was more severe than the disease occurring in non-vaccinated controls (Grayston and Wang 1978). The fact that the initial trials with 10 inactivated whole organisms resulted in some cases of what appeared to be a hypersensitivity reaction prompted attempts to develop subunit vaccines.

C. trachomatis holds, as well as secretes, several proteins of potential relevance for the generation of a chlamydia vaccine. For a number of years, the search for candidate 15 molecules has primarily focused on proteins associated with the surface of the infectious form the Elementary Body (EB). Despite the characterization of a large number of such proteins only a few of these have been demonstrated to elicit partial protection as subunit vaccines in animal models. The first immunogenic molecule described was the major outer membrane protein (MOMP), and this molecule has therefore been studied in great detail as 20 a candidate vaccine. However, many attempts to immunize different animals with MOMP extracted from *C. trachomatis* or recombinant preparations gave variable results (Su, Parnell et al. 1995; Pal, Barnhart et al. 1999; Zhang, Yang et al. 1999; Pal, Theodor et al. 2001; Shaw, Grund et al. 2002). The reason for the relative ineffectiveness of MOMP as a vaccine is not known, but may result from inadequate adjuvants or delivery systems or 25 from use of MOMP immunogens that do not mimic the native structure of the protein (Pal, Theodor et al. 2001)

More recently, several other immunogenic molecules have been identified (Hassell, 30 Reynolds et al. 1993; Kubo and Stephens 2000; LaVerda, Albanese et al. 2000; Fling, Sutherland et al. 2001; Goodall, Yeo et al. 2001; Starnbach, Loomis et al. 2003).

Immunity to *C. trachomatis* is characterized by some basic features; specifically sensitized T lymphocytes mediates protection (Su and Caldwell 1995; Morrison, Su et al. 2000; Morrison and Caldwell 2002), and the most important mediator molecule seems to be interferon gamma (IFN γ) (Morrison and Caldwell 2002). Additionally antibodies of the
5 IgG, IgM, and IgA isotypes may also play a role (Cotter, Meng et al. 1995).
In 1995 Tripples et al. (Tripples and McClarty 1995) isolated the gene for the CTP synthetase and Gu et al. (Gu, Wenman et al. 1995) cloned the region surrounding the gene for the alpha subunit of RNA polymerase. This region also contains genes for the proteins SecY, S13, S11, and L17, which are equivalent to *Escherichia coli* and *Bacillus subtilis*
10 proteins. In 1997, the gene for elongation factor Ts was isolated (Zhang, Tao et al. 1997).

In 1998 Stevens et al published the complete genome sequence of *C. trachomatis* and predicted the presence of approximately 875 open reading frames. Among others, nucleotide sequences comprising CT442, CT460, CT509 CT579, CT587, CT713, CT812,
15 or CT681 (MOMP) are described, and putative protein sequences for the above sequences are suggested. However importantly, this sequence information cannot be used to predict if the DNA is transcribed and translated into proteins *in vivo*.

More importantly, it is not possible on the basis of the sequences, to predict whether a
20 given sequence will encode an immunogenic or an inactive protein. WO9928475 describes the complete genome sequence of *C. trachomatis* but has no evidence in support of any immunogenic effect whatsoever. Correspondingly WO9927105 describes the complete genome sequence of *C. pneumoniae*.

25 The only way to determine if a protein is recognized by the immune system during or after an infection with *C. trachomatis* is to produce the given protein and test it in an appropriate assay as described herein and possibly determine the fragment or epitope that has an immunogenic effect.

Summary of the invention

The invention is related to prevention, treatment and detection of infections caused by Chlamydia species (*C. trachomatis ssp* and *C. pneumonia*) by the use of a polypeptide
 5 comprising a *C. trachomatis* antigen or an immunogenic portion or other variant thereof, or by the use of a DNA sequence encoding a *C. trachomatis* antigen or an immunogenic portion or other variant thereof.

Detailed disclosure of the invention

10 The present invention discloses the use of the Chlamydia antigens (polypeptides or nucleic acids) ct043, ct511, ct521, ct616, ct803, ct067, ct679, ct583, ct603, ct026, ct093, ct357, ct659, ct111, ct509, ct587, ct023, ct025, ct078, ct082, ct118, ct174, ct003, ct005, ct027, ct032, ct008, ct016, ct028, ct035, ct141, ct643, ct414, ct874, ct456, ct681, ct123, ct125, ct126, ct133, ct150, ct175, ct376, ct083, ct089, ct155, ct168, ct175, ct184, ct124, ct082,
 15 ct336, ct342, ct842, ct323, ct080, ct084, ct110, ct119, ct541, ct443, ct795, ct396, ct283, ct051, ct002, ct009, ct015, ct030, ct048, ct061, ct063, ct068, ct071, ct051, ct080, ct115, ct119, ct678, ct561, ct538, ct582, ct875, ct322, ct112, ct315, ct610, , ct147, ct228, ct232, ct614, ct098, ct265, ct375, ct004, ct038, ct040, ct052, ct053, ct201, ct245, ct246, ct405, ct420, ct426, ct507, ct512, ct513, ct514, ct516, ct316, ct439, ct492, ct520, ct523, ct526,
 20 ct611, ct613, ct626, ct630, ct647, ct649, ct725, ct734, ct779, ct801, ct833, ct835, ct836, ct845 or fragments (immunogenic portion, e.g. a T-cell or B-cell epitope) or homologs hereof for preparation of a pharmaceutical composition for preventing, treating or diagnosing infections caused by a bacteria from the Chlamydia species.

25 The invention also discloses the use of specific peptide fragments e.g. CT541-PF1 (aa pos. 111-243), CT443-PF1 (aa pos. 214-291), CT795-PF1 (aa pos. 1-163), CT396-PF1 (aa pos. 170-318), CT842-PF1 (aa pos. 433-515), CT283-PF1 (aa pos. 477-577), CT874-PF1 (aa pos. 330-426), CT051-PF1 (aa pos. 38-177), CT141-PF1 (aa pos. 17-126), CT643-PF1 (aa pos. 769-841), CT681-PF1 (aa pos. 156-391), CT681-PF2 (aa pos. 199-329), CT681-PF3
 30 (aa pos. 294-349), CT414-PF1 (aa pos. 605-722), CT414-PF2 (aa pos. 463-530), CT456-PF1 (aa pos. 695-840), CT456-PF2 (aa pos. 137-229), CT456-PF3 (aa pos. 243-321), CT456-PF4 (aa pos. 209-291), CT456-PF5 (aa pos. 175-279), CT456-PF6 (aa pos. 567-

730), CT456-PF7 (aa pos. 210-540), CT456-PF8 (aa pos. 190-279), CT521-PF1 (aa pos. 14-36), CT521-PF2 (aa pos. 40-62), CT521-PF3 (aa pos. 52-75), CT521-PF4 (aa pos. 66-88), CT521-PF5 (aa pos. 116-138), CT504-PF1 or the nucleic acid encoding these peptide fragments, for preparation of a pharmaceutical composition for preventing, treating or
5 diagnosing infections caused by a bacteria from the Chlamydia species.

The present invention also discloses a pharmaceutical composition in the form of a vaccine or a diagnostic agent

10 The polypeptide used for preparation of the pharmaceutical composition can be lipidated to allow a self- adjuvating effect or fused to a fusion partner where the fusion partner can be another polypeptide derived from *C. trachomatis*, including, but not limited to, one or more polypeptide fragments derived from CT812, CT579, CT587, Cap, CT713, CT442 or MOMP or at least one T-cell or B-cell epitope of any of the above mentioned. The
15 invention also pertains to a fusion polypeptide comprising mutual fusions of two or more of the polypeptides (or immunogenic portions thereof) of the invention.

The vaccine disclosed by the invention can be used for preventing or treating an infection of the Chlamydia species, e.g. *C. trachomatis*.

20

The diagnostic agent disclosed by the invention (above mentioned antigen or an antibody against it) can be used for diagnosis of an infection of the Chlamydia species, e.g. *C. trachomatis*.

25 The diagnostic methods disclosed are based on cell mediated immunity, serology or a simple skin test. Diagnosis by cell mediated immunity of previous or ongoing infection with a bacterium from the Chlamydia species, comprises contacting a sample, e.g. a blood sample comprising mononuclear cells (e.g. T-lymphocytes), with the diagnostic reagent in order to detect a positive reaction, e.g. proliferation of the cells or release of cytokines
30 such as IFN γ . Diagnosis by serology of previous or ongoing infection with a bacterium from the Chlamydia species said method comprising contacting a sample, e.g. a blood sample, with an antibody against the antigen in order to detect a positive reaction in case of

infection or by contacting the antigen with a bodily fluid of the subject and when detecting binding of an antibody to said polypeptide, said binding being an indication that said subject is infected by a bacterium from the Chlamydia species. A skin test comprises intradermally injecting or applying to the skin, e.g. by a patch, the diagnostic reagent, a
5 positive skin response at the location of injection or applying being indicative of an infection with a bacterium from the Chlamydia species.

The present invention also discloses a method for immunizing against an infection of a bacterium from the Chlamydia species, comprising administering the above mentioned
10 vaccine of the invention to a mammal.

Definitions

Polypeptides

The word "polypeptide" in the present invention should have its usual meaning. That is an
15 amino acid chain of any length, including a full-length protein, oligopeptides, short peptides and fragments thereof, wherein the amino acid residues are linked by covalent peptide bonds.

The polypeptide may be chemically modified by being glycosylated, by being lipidated
20 (e.g. by chemical lipidation with palmitoyloxy succinimide as described by Mowat et al. 1991 or with dodecanoyl chloride as described by Lustig et al. 1976), by comprising prosthetic groups, or by containing additional amino acids such as e.g. a his-tag or a signal peptide.

25 Each polypeptide may thus be characterised by specific amino acids and be encoded by specific nucleic acid sequences. It will be understood that such sequences include analogues and variants produced by recombinant or synthetic methods wherein such polypeptide sequences have been modified by substitution, insertion, addition or deletion of one or more amino acid residues in the recombinant polypeptide and still be
30 immunogenic in any of the biological assays described herein. Substitutions are preferably "conservative". These are defined according to the following table. Amino acids in the

same block in the second column and preferably in the same line in the third column may be substituted for each other. The amino acids in the third column are indicated in one-letter code.

ALIPHATIC	Non-polar	GAP
		ILV
	Polar-uncharged	CSTM
		NQ
	Polar-charged	DE
		KR
AROMATIC		HFYW

- 5 A preferred polypeptide within the present invention is an immunogenic antigen from *C. trachomatis*. Such antigen can for example be derived from the *C. trachomatis* cell and/or *C. trachomatis* culture filtrate. Thus, a polypeptide comprising an immunogenic portion of one of the above antigens may consist entirely of the immunogenic portion, or may contain additional sequences. The additional sequences may be derived from the native *C.*
- 10 *trachomatis* antigen or be heterologous and such sequences may, but need not, be immunogenic.

Each polypeptide is encoded by a specific nucleic acid sequence. It will be understood that such sequences include analogues and variants hereof wherein such nucleic acid sequences

15 have been modified by substitution, insertion, addition or deletion of one or more nucleic acid. Substitutions are preferably silent substitutions in the codon usage which will not lead to any change in the amino acid sequence, but may be introduced to enhance the expression of the protein.

- 20 In the present context the term "substantially pure polypeptide fragment" means a polypeptide preparation which contains at most 5% by weight of other polypeptide material with which it is natively associated (lower percentages of other polypeptide material are preferred, e.g. at most 4%, at most 3%, at most 2%, at most 1%, and at most

½%). It is preferred that the substantially pure polypeptide is at least 96% pure, *i.e.* that the polypeptide constitutes at least 96% by weight of total polypeptide material present in the preparation, and higher percentages are preferred, such as at least 97%, at least 98%, at least 99%, at least 99,25%, at least 99,5%, and at least 99,75%. It is especially preferred
5 that the polypeptide fragment is in "essentially pure form", *i.e.* that the polypeptide fragment is essentially free of any other antigen with which it is natively associated, *i.e.* free of any other antigen from bacteria belonging to the *Chlamydia* species. This can be accomplished by preparing the polypeptide fragment by means of recombinant methods in a non-chlamydia host cell as will be described in detail below, or by synthesizing the
10 polypeptide fragment by the well-known methods of solid or liquid phase peptide synthesis, e.g. by the method described by Merrifield or variations thereof.

By the term " *Chlamydia* species " is understood a bacterium capable of causing the *Chlamydia* infection in an animal or in a human being. Examples are *C. trachomatis*, *C.*
15 *pneumoniae* and *C. muridarum*.

The Major Outer Membrane Protein (MOMP) of *C. trachomatis*, is expressed during all phases of the developmental life cycle of *C. trachomatis* and constitute approximately 60% of the total protein content of the chlamydia outer membrane. MOMP can be divided into
20 conserved domains interrupted by four highly variable domains (VD1-4) (Stephens, Wagar et al. 1988). In general T cell epitopes are located in the conserved regions (Ortiz, Demick et al. 1996) whereas the human antibody response is primarily directed against the variable domains. Based on the reactivity of specific mono clonal antibodies and detailed sequence analysis of the variable regions *C. trachomatis* can be divided into 15 different
25 serovariants and of these serovariants A, B, Ba and C causes Trachoma, D – K causes sexually transmitted disease (STD), L1 - L3 causes Lymphogranuloma venerum, and MoPn (*C. muridarum*) infects mice.

By "a *Chlamydia* patient" is understood an individual with culture or PCR proven infection
30 with *Chlamydia* spp. Culture, microscopy and PCR diagnosis of *Chlamydia* are well known by any person skilled in the art.

By the term "delayed type hypersensitivity reaction" (DTH) is understood a T-cell mediated inflammatory response elicited after the injection of a polypeptide into, or application to, the skin, said inflammatory response appearing 72-96 hours after the polypeptide injection or application.

5

By the term "IFN γ " is understood interferon-gamma. The measurement of IFN γ is used as an indication of an immunological response.

By the terms "nucleic acid fragment" and "nucleic acid sequence" are understood any nucleic acid molecule including DNA, RNA, LNA (locked nucleic acids), PNA, RNA, dsRNA and RNA-DNA-hybrids. Also included are nucleic acid molecules comprising non-naturally occurring nucleosides. The term includes nucleic acid molecules of any length e.g. from 10 to 10000 nucleotides, depending on the use. When the nucleic acid molecule is for use as a pharmaceutical, e.g. in DNA therapy, or for use in a method for producing a polypeptide according to the invention, a molecule encoding at least one epitope is preferably used, having a length from about 18 to about 1000 nucleotides, the molecule being optionally inserted into a vector. When the nucleic acid molecule is used as a probe, as a primer or in antisense therapy, a molecule having a length of 10-100 is preferably used. According to the invention, other molecule lengths can be used, for instance a molecule having at least 12, 15, 21, 24, 27, 30, 33, 36, 39, 42, 50, 60, 70, 80, 90, 100, 200, 300, 400, 500 or 1000 nucleotides (or nucleotide derivatives), or a molecule having at most 10000, 5000, 4000, 3000, 2000, 1000, 700, 500, 400, 300, 200, 100, 50, 40, 30 or 20 nucleotides (or nucleotide derivatives).

25

The term "stringent" when used in conjunction with hybridization conditions is as defined in the art, i.e. the hybridization is performed at a temperature not more than 15-20°C under the melting point T_m , cf. Sambrook et al, 1989, pages 11.45-11.49. Preferably, the conditions are "highly stringent", i.e. 5-10°C under the melting point T_m .

30

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations thereof such as "comprises" or "comprising", will be understood to imply the

inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

Sequence identity

The term "sequence identity" indicates a quantitative measure of the degree of homology between two amino acid sequences of equal length or between two nucleotide sequences of equal length. The two sequences to be compared must be aligned to best possible fit possible with the insertion of gaps or alternatively, truncation at the ends of the protein sequences. The sequence identity can be calculated as $\frac{(N_{ref}-N_{dif})}{N_{ref}} \times 100$, wherein N_{dif} is the total number of non-identical residues in the two sequences when aligned and wherein N_{ref} is the number of residues in one of the sequences. Hence, the DNA sequence AGTCAGTC will have a sequence identity of 75% with the sequence AATCAATC ($N_{dif}=2$ and $N_{ref}=8$). A gap is counted as non-identity of the specific residue(s), i.e. the DNA sequence AGTGTC will have a sequence identity of 75% with the DNA sequence AGTCAGTC ($N_{dif}=2$ and $N_{ref}=8$). Sequence identity can alternatively be calculated by the BLAST program e.g. the BLASTP program (Pearson and Lipman 1988)

In one aspect of the invention, alignment is performed with the sequence alignment method ClustalW with default parameters as described by Thompson J., *et al* 1994.

A preferred minimum percentage of sequence identity is at least 80%, such as at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, and at least 99.5%.

Immunogenic portion

In a preferred embodiment of the invention, the polypeptide comprises an immunogenic portion of the polypeptide, such as an epitope for a B-cell or T-cell.

The immunogenic portion of a polypeptide is a part of the polypeptide, which elicits an immune response in an animal or a human being, and/or in a biological sample determined by any of the biological assays described herein. The immunogenic portion of a polypeptide may be a T-cell epitope or a B-cell epitope. Immunogenic portions can be related to one or a few relatively small parts of the polypeptide, they can be scattered throughout the polypeptide sequence or be situated in specific parts of the polypeptide. For

a few polypeptides epitopes have even been demonstrated to be scattered throughout the polypeptide covering the full sequence (Ravn, Demissie et al. 1999).

In order to identify relevant T-cell epitopes which are recognised during an immune response, it is possible to use a "brute force" method: Since T-cell epitopes are linear,

5 deletion mutants of the polypeptide will, if constructed systematically, reveal what regions of the polypeptide are essential in immune recognition, e.g. by subjecting these deletion mutants e.g. to the IFN γ assay described herein. Another method utilises overlapping oligopeptides for the detection of MHC class II epitopes, preferably synthetic, having a length of e.g. 20 amino acid residues derived from the polypeptide. These peptides can be
10 tested in biological assays (e.g. the IFN γ assay as described herein) and some of these will give a positive response (and thereby be immunogenic) as evidence for the presence of a T cell epitope in the peptide. For the detection of MHC class I epitopes it is possible to predict peptides that will bind (Stryhn, Pedersen et al. 1996) and hereafter produce these peptides synthetic and test them in relevant biological assays e.g. the IFN γ assay as
15 described herein. The peptides preferably having a length of e.g. 8 to 11 amino acid residues derived from the polypeptide. B-cell epitopes can be determined by analysing the B cell recognition to overlapping peptides covering the polypeptide of interest as e.g. described in Harboe et al (Harboe, Oettinger et al. 1996).

20 Although the minimum length of a T-cell epitope has been shown to be at least 6 amino acids, it is normal that such epitopes are constituted of longer stretches of amino acids. Hence, it is preferred that the polypeptide fragment of the invention has a length of at least 7 amino acid residues, such as at least 8, at least 9, at least 10, at least 12, at least 14, at least 16, at least 18, at least 20, at least 22, at least 24, and at least 30 amino acid residues.
25 Hence, in important embodiments of the inventive method, it is preferred that the polypeptide fragment has a length of at most 50 amino acid residues, such as at most 40, 35, 30, 25, and 20 amino acid residues. It is expected that the peptides having a length of between 10 and 20 amino acid residues will prove to be most efficient as MCH class II epitopes and therefore especially preferred lengths of the polypeptide fragment used in the
30 inventive method are 18, such as 15, 14, 13, 12 and even 11 amino acid residues. It is expected that the peptides having a length of between 7 and 12 amino acid residues will prove to be most efficient as MCH class I epitopes and therefore especially preferred

lengths of the polypeptide fragment used in the inventive method are 11, such as 10, 9, 8 and even 7 amino acid residues.

Immunogenic portions of polypeptides may be recognised by a broad part (high frequency) or by a minor part (low frequency) of the genetically heterogenic human population. In addition some immunogenic portions induce high immunological responses (dominant), whereas others induce lower, but still significant, responses (subdominant). High frequency > low frequency can be related to the immunogenic portion binding to widely distributed MHC molecules (HLA type) or even by multiple MHC molecules (Kilgus, Jardetzky et al. 1991) (Sinigaglia, Guttinger et al. 1988).

In the context of providing candidate molecules for a new vaccine against Chlamydia infection, the subdominant epitopes are however as relevant as are the dominant epitopes since it has been shown that such epitopes can induce protection regardless of being subdominant.

Variants

A common feature of the polypeptides of the invention is their capability to induce an immunological response as illustrated in the examples. It is understood that a variant of a polypeptide of the invention produced by substitution, insertion, addition or deletion is also immunogenic determined by any of the assays described herein.

Immune individual

An immune individual is defined as a person or an animal, which has cleared or controlled an infection with chlamydia.

Immunogenic

An immunogenic polypeptide is defined as a polypeptide that induces an immune response in a biological sample or an individual currently or previously infected with a chlamydia.

The immune response may be monitored by one of the following methods:

- An in vitro cellular response is determined by release of a relevant cytokine such as IFN γ , from lymphocytes withdrawn from an animal or human being currently or previously infected with chlamydia, or by detection of proliferation of these T cells. The induction being performed by the addition of the polypeptide or the immunogenic portion to a suspension comprising from 1×10^5 cells to 3×10^5 cells per well. The cells being isolated from either the blood, the spleen, the liver or the lung and the addition of the polypeptide or the immunogenic portion resulting in a concentration of not more than 20 μ g per ml suspension and the stimulation being performed from two to five days. For monitoring cell proliferation the cells are pulsed with radioactive labeled Thymidine and after 16-22 hours of incubation detecting the proliferation by liquid scintillation counting. A positive response being a response more than background plus two standard deviations. The release of IFN γ can be determined by the ELISA method, which is well known to a person skilled in the art. A positive response being a response more than background plus two standard deviations. Other cytokines than IFN γ could be relevant when monitoring the immunological response to the polypeptide, such as IL-12, TNF- α , IL-4, IL-5, IL-10, IL-6, TGF- β . Another and more sensitive method for determining the presence of a cytokine (e.g. IFN γ) is the ELISPOT method where the cells isolated from either the blood, the spleen, the liver or the lung are diluted to a concentration of preferable of 1 to 4×10^6 cells /ml and incubated for 18-22 hrs in the presence of of the polypeptide or the immunogenic portion resulting in a concentration of not more than 20 μ g per ml. The cell suspensions are hereafter diluted to 1 to 2×10^6 / ml and transferred to Maxisorp plates coated with anti-IFN γ and incubated for preferably 4 to 16 hours. The IFN γ producing cells are determined by the use of labelled secondary anti-IFN γ antibody and a relevant substrate giving rise to spots, which can be enumerated using a dissection microscope. It is also a possibility to determine the presence of mRNA coding for the relevant cytokine by the use of the PCR technique. Usually one or more cytokines will be measured utilizing for example the PCR, ELISPOT or ELISA. It will be appreciated by a person skilled in the art that a significant increase or decrease in the amount of any of these cytokines induced by a specific polypeptide can be used in evaluation of the immunological activity of the polypeptide.

- An *in vitro* cellular response may also be determined by the use of T cell lines derived from an immune individual or a *C. trachomatis* infected person where the T cell lines have been driven with either live chlamydia or extracts from the bacterial cell for 10 to 20 days with the addition of IL-2. The induction being performed by addition of not more than 20 µg polypeptide per ml suspension to the T cell lines containing from 1×10^5 cells to 3×10^5 cells per well and incubation being performed from two to six days. The induction of IFN γ or release of another relevant cytokine is detected by ELISA. The stimulation of T cells can also be monitored by detecting cell proliferation using radioactively labeled Thymidine as described above. For both assays a positive response being a response more than background plus two standard deviations.
- An *in vivo* cellular response which may be determined as a positive DTH response after intradermal injection or local application patch of at most 100 µg of the polypeptide or the immunogenic portion to an individual who is clinically or subclinically infected with chlamydia, a positive response having a diameter of at least 5 mm 72-96 hours after the injection or application.
- An *in vitro* humoral response is determined by a specific antibody response in an immune or infected individual. The presence of antibodies may be determined by an ELISA technique or a Western blot where the polypeptide or the immunogenic portion is absorbed to either a nitrocellulose membrane or a polystyrene surface. The serum is preferably diluted in PBS from 1:10 to 1:100 and added to the absorbed polypeptide and the incubation being performed from 1 to 12 hours. By the use of labeled secondary antibodies the presence of specific antibodies can be determined by measuring the OD e.g. by ELISA where a positive response is a response of more than background plus two standard deviations or alternatively a visual response in a Western blot.
- Another relevant parameter is measurement of the protection in animal models

induced after vaccination with the polypeptide in an adjuvant or after DNA vaccination. Suitable animal models include primates, guinea pigs or mice, which are challenged with an infection of chlamydia. Readout for induced protection could be decrease of the bacterial load in target organs compared to non-vaccinated animals, prolonged survival times compared to non-vaccinated animals and diminished weight loss compared to non-vaccinated animals.

Preparation methods

In general, *C. trachomatis* antigens, and DNA sequences encoding such antigens, may be prepared using any one of a variety of procedures.

They may be purified as native proteins from the *C. trachomatis* cell by procedures such as those described above. Immunogenic antigens may also be produced recombinantly using a DNA sequence encoding the antigen, which has been inserted into an expression vector and expressed in an appropriate host. Examples of host cells are *E. coli*. The polypeptides or immunogenic portion hereof can also be produced synthetically having fewer than about 100 amino acids, and generally fewer than 50 amino acids and may be generated using techniques well known to those ordinarily skilled in the art, such as commercially available solid-phase techniques where amino acids are sequentially added to a growing amino acid chain.

20

In the construction and preparation of plasmid DNA encoding the polypeptide as defined for DNA vaccination a host strain such as *E. coli* can be used. Plasmid DNA can then be prepared from overnight cultures of the host strain carrying the plasmid of interest, and purified using e.g. the Qiagen Giga -Plasmid column kit (Qiagen, Santa Clarita, CA, USA) including an endotoxin removal step. It is essential that plasmid DNA used for DNA vaccination is endotoxin free.

25

Fusion proteins

The immunogenic polypeptides may also be produced as fusion proteins, by which methods superior characteristics of the polypeptide of the invention can be achieved. For instance, fusion partners that facilitate export of the polypeptide when produced recombinantly, fusion partners that facilitate purification of the polypeptide, and fusion partners

30

which enhance the immunogenicity of the polypeptide fragment of the invention are all interesting possibilities. Therefore, the invention also pertains to a fusion polypeptide comprising at least one polypeptide or immunogenic portion defined above and at least one fusion partner. The fusion partner can, in order to enhance immunogenicity, be another polypeptide derived from *C. trachomatis*, such as a polypeptide fragment derived from Chlamydia species, such as CT812, CT579, CT587 (Goodall, Yeo et al. 2001), Cap (Fling, Sutherland et al. 2001), CT713 (Kubo and Stephens 2000), CT442 (Starnbach, Loomis et al. 2003), or MOMP (Stephens, Wagar et al. 1988) or at least one T-cell epitope or B cell epitope of any of the above mentioned. The invention also pertains to a fusion polypeptide comprising mutual fusions of two or more of the polypeptides (or immunogenic portions thereof) of the invention.

Other fusion partners, which could enhance the immunogenicity of the product, are lymphokines such as IFN γ , IL-2 and IL-12. In order to facilitate expression and/or purification, the fusion partner can e.g. be a bacterial fimbrial protein, e.g. the pilus components pilin and papA; protein A; the ZZ-peptide (ZZ-fusions are marketed by Pharmacia in Sweden); the maltose binding protein; glutathione S-transferase; β -galactosidase; or poly-histidine. Fusion proteins can be produced recombinantly in a host cell, which could be *E. coli*, and it is a possibility to induce a linker region between the different fusion partners.

Other interesting fusion partners are polypeptides, which are lipidated so that the immunogenic polypeptide is presented in a suitable manner to the immune system. This effect is e.g. known from vaccines based on the *Borrelia burgdorferi* OspA polypeptide as described in e.g. WO 96/40718 A or vaccines based on the *Pseudomonas aeruginosa* OprI lipoprotein (Cote-Sierra, Jongert et al. 1998). Another possibility is N-terminal fusion of a known signal sequence and an N-terminal cysteine to the immunogenic polypeptide. Such a fusion results in lipidation of the immunogenic polypeptide at the N-terminal cysteine, when produced in a suitable production host.

30

Pharmaceutical composition

A pharmaceutical composition is defined as any vaccine (both therapeutic and

prophylactic) or any diagnostic reagent as described in the following.

Vaccine, protein

Another part of the invention pertains to a vaccine composition comprising a polypeptide
5 (or at least one immunogenic portion thereof) or fusion polypeptide according to the invention. In order to ensure optimum performance of such a vaccine composition it is preferred that it comprises an immunologically and pharmaceutically acceptable carrier, vehicle or adjuvant.

- 10 An effective vaccine, wherein a polypeptide of the invention is recognized by the animal, will in an animal model be able to decrease bacterial load in target organs, prolong survival times and/or diminish weight loss after challenge with virulent Chlamydia, compared to non-vaccinated animals
- 15 Suitable carriers are selected from the group consisting of a polymer to which the polypeptide(s) is/are bound by hydrophobic non-covalent interaction, such as a plastic, e.g. polystyrene, or a polymer to which the polypeptide(s) is/are covalently bound, such as a polysaccharide, or a polypeptide, e.g. bovine serum albumin, ovalbumin or keyhole limpet haemocyanin. Suitable vehicles are selected from the group consisting of a diluent and a
20 suspending agent. The adjuvant is preferably selected from the group consisting of dimethyldioctadecylammonium bromide (DDA), Quil A, poly I:C, aluminium hydroxide, Freund's incomplete adjuvant, IFN γ , IL-2, IL-12, monophosphoryl lipid A (MPL), Trehalose Dimycolate (TDM), Trehalose Dibehenate (TDB) and muramyl dipeptide (MDP).

25

Preparation of vaccines which contain peptide sequences as active ingredients is generally well understood in the art, as exemplified by U.S. Patents 4,608,251; 4,601,903; 4,599,231 and 4,599,230.

- 30 Other methods of achieving adjuvant effect for the vaccine include use of agents such as aluminum hydroxide or phosphate (alum), synthetic polymers of sugars (Carbopol), aggregation of the protein in the vaccine by heat treatment, aggregation by reactivating

with pepsin treated (Fab) antibodies to albumin, mixture with bacterial cells such as *C. parvum* or endotoxins or lipopolysaccharide components of gram-negative bacteria, emulsion in physiologically acceptable oil vehicles such as mannide mono-oleate (Aracel A) or emulsion with 20 percent solution of a perfluorocarbon (Fluosol-DA) used as a block
5 substitute may also be employed. Other possibilities involve the use of immune modulating substances such as cytokines or synthetic IFN γ inducers such as poly I:C in combination with the above-mentioned adjuvants.

Another interesting possibility for achieving adjuvant effect is to employ the technique described in (Gosselin, Wardwell et al. 1992).
10

In brief, a relevant antigen such as an antigen of the present invention can be conjugated to an antibody (or antigen binding antibody fragment) against the Fc γ receptors on monocytes/macrophages.

15 The vaccines are administered in a manner compatible with the dosage formulation, and in such amount as will be therapeutically effective and immunogenic. The quantity to be administered depends on the subject to be treated, including, e.g., the capacity of the individual's immune system to mount an immune response, and the degree of protection desired. Suitable dosage ranges are of the order of several hundred micrograms active
20 ingredient per vaccination with a preferred range from about 0.1 μ g to 1000 μ g, such as in the range from about 1 μ g to 300 μ g, and especially in the range from about 10 μ g to 50 μ g. Suitable regimens for initial administration and booster shots are also variable but are typified by an initial administration followed by subsequent inoculations or other administrations.

25

The manner of application may be varied widely. Any of the conventional methods for administration of a vaccine are applicable. These are believed to include oral application on a solid physiologically acceptable base or in a physiologically acceptable dispersion, parenterally, by injection or the like. The dosage of the vaccine will depend on the route of
30 administration and will vary according to the age of the person to be vaccinated and, to a lesser degree, the size of the person to be vaccinated.

The vaccines are conventionally administered parenterally, by injection, for example, either subcutaneously or intramuscularly. Additional formulations which are suitable for other modes of administration include suppositories and, in some cases, oral formulations. For suppositories, traditional binders and carriers may include, for example, polyalkalene glycols or triglycerides; such suppositories may be formed from mixtures containing the active ingredient in the range of 0.5% to 10%, preferably 1-2%. Oral formulations include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, and the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and advantageously contain 10-95% of active ingredient, preferably 25-70%.

In many instances, it will be necessary to have multiple administrations of the vaccine. Especially, vaccines can be administered to prevent an infection with chlamydia and/or to treat established chlamydia infection. When administered to prevent an infection, the vaccine is given prophylactically, before definitive clinical signs or symptoms of an infection are present.

Due to genetic variation, different individuals may react with immune responses of varying strength to the same polypeptide. Therefore, the vaccine according to the invention may comprise several different polypeptides in order to increase the immune response. The vaccine may comprise two or more polypeptides or immunogenic portions, where all of the polypeptides are as defined above, or some but not all of the peptides may be derived from one or more of the other chlamydia serovariants. In the latter example, the polypeptides not necessarily fulfilling the criteria set forth above for polypeptides may either act due to their own immunogenicity or merely act as adjuvants.

The vaccine may comprise 1-20, such as 2-20 or even 3-20 different polypeptides or fusion polypeptides, such as 3-10 different polypeptides or fusion polypeptides.

The invention also pertains to a method for immunising an animal, including a human being, against Chlamydia infection caused by a Chlamydia species, comprising administering to the animal the polypeptide of the invention, or a vaccine composition of

the invention as described above, or a living vaccine described above.

The invention also pertains to a method for producing an immunologic composition according to the invention, the method comprising preparing, synthesising or isolating a
5 polypeptide according to the invention, and solubilizing or dispersing the polypeptide in a medium for a vaccine, and optionally adding other *C. trachomatis* antigens and/or a carrier, vehicle and/or adjuvant substance.

Vaccine DNA.

The nucleic acid fragments of the invention may be used for effecting *in vivo* expression of
10 antigens, *i.e.* the nucleic acid fragments may be used in so-called DNA vaccines as reviewed in (Ulmer, Donnelly et al. 1993), which is included by reference.

Hence, the invention also relates to a vaccine comprising a nucleic acid fragment according to the invention, the vaccine effecting *in vivo* expression of antigen by an animal,
15 including a human being, to whom the vaccine has been administered, the amount of expressed antigen being effective to confer substantially increased resistance to infections caused by virulent *chlamydia* in an animal, including a human being.

The efficacy of such a DNA vaccine can possibly be enhanced by administering the gene
20 encoding the expression product together with a DNA fragment encoding a polypeptide which has the capability of modulating an immune response.

Live recombinant vaccines

One possibility for effectively activating a cellular immune response for a vaccine can be
25 achieved by expressing the relevant antigen in a vaccine in a non-pathogenic microorganism or virus. Well-known examples of such microorganisms are *Mycobacterium bovis* BCG, *Salmonella* and *Pseudomona* and examples of viruses are Vaccinia Virus and Adenovirus.

30 Another possibility is to integrate the DNA encoding the polypeptide according to the invention in an attenuated virus such as the vaccinia virus or Adenovirus (Rolph and Ramshaw 1997). The recombinant vaccinia virus is able to replicate within the cytoplasm

of the infected host cell and the polypeptide of interest can therefore induce an immune response, which is envisioned to induce protection against Chlamydia.

Therapeutic vaccine.

The invention also relates to the use of a polypeptide or nucleic acid of the invention for
5 use as therapeutic vaccines as have been described in the literature exemplified by D.
Lowry (Lowry et al 1999). Antigens with therapeutic properties may be identified based
on their ability to diminish the severity of *C. trachomatis* infection in experimental animals
or prevent reactivation of previous infection, when administered as a vaccine. The
composition used for therapeutic vaccines can be prepared as described above for
10 vaccines.

Diagnostic protein

The invention also relates to a method of diagnosing Chlamydia infection caused by a
chlamydia in an animal, including a human being, comprising intradermally injecting, in
the animal, a polypeptide according to the invention, a positive skin response at the
15 location of injection being indicative of the animal having a Chlamydia infection, and a
negative skin response at the location of injection being indicative of the animal not having
an infection.

When diagnosis of previous or ongoing infection with virulent chlamydia is the aim, a
20 blood sample comprising mononuclear cells (*i.e.* T-lymphocytes) from a patient could be
contacted with a sample of one or more polypeptides of the invention. This contacting can
be performed *in vitro* and a positive reaction could e.g. be proliferation of the T-cells or
release of cytokines such as IFN γ into the extracellular phase. It is also conceivable to
contact a serum sample from a subject with a polypeptide of the invention, the demonstra-
25 tion of a binding between antibodies in the serum sample and the polypeptide being indica-
tive of previous or ongoing infection.

The invention therefore also relates to an *in vitro* method for diagnosing ongoing or
previous sensitisation in an animal or a human being with a Chlamydia species, the method
30 comprising providing a blood sample from the animal or human being, and contacting the
sample from the animal with the polypeptide of the invention, a significant release into the

extracellular phase of at least one cytokine by mononuclear cells in the blood sample being indicative of the animal being sensitised. A positive response being a response more than release from a blood sample derived from a patient without the chlamydia diagnosis plus two standard deviations. The invention also relates to the *in vitro* method for diagnosing ongoing or previous sensitisation in an animal or a human being with chlamydia, the method comprising providing a blood sample from the animal or human being, and by contacting the sample from the animal with the polypeptide of the invention demonstrating the presence of antibodies recognizing the polypeptide of the invention in the serum sample. The immunogenic composition used for diagnosing may comprise 1-20, such as 2-20 or even 3-20 different polypeptides or fusion polypeptides, such as 3-10 different polypeptides or fusion polypeptides.

Diagnostic DNA

The nucleic acid probes encoding the polypeptide of the invention can be used in a variety of diagnostic assays for detecting the presence of pathogenic organisms in a given sample. A method of determining the presence of chlamydial nucleic acids in an animal, including a human being, or in a sample, comprising administering a nucleic acid fragment of the invention to the animal or incubating the sample with the nucleic acid fragment of the invention or a nucleic acid fragment complementary thereto, and detecting the presence of hybridised nucleic acids resulting from the incubation (by using the hybridisation assays which are well-known in the art), is also included in the invention. Such a method of diagnosing Chlamydia infection might involve the use of a composition comprising at least a part of a nucleotide sequence as defined above and detecting the presence of nucleotide sequences in a sample from the animal or human being to be tested which hybridise with the nucleic acid fragment (or a complementary fragment) by the use of PCR technique.

Antibodies

A monoclonal or polyclonal antibody, which is specifically reacting with a polypeptide of the invention in an immuno assay, or a specific binding fragment of said antibody, is also a part of the invention. The antibodies can be produced by methods known to the person skilled in the art. Polyclonal antibodies can be raised in a mammal, for example, by one or more injections of a polypeptide according to the present invention and, if desired, an adjuvant. The monoclonal antibodies according to the present invention may, for example,

be produced by the hybridoma method first described by Kohler and Milstein (1975), or may be produced by recombinant DNA methods such as described in U.S. Pat. No. 4,816,567. The monoclonal antibodies may also be isolated from phage libraries generated using the techniques described by (McCafferty, Griffiths et al. 1990), for example.

5 Methods for producing antibodies are described in the literature, e.g. in US6136958.

A sample of a potentially infected organ may be contacted with such an antibody recognizing a polypeptide of the invention. The demonstration of the reaction by means of methods well known in the art between the sample and the antibody will be indicative of
 10 an ongoing infection. It is of course also a possibility to demonstrate the presence of anti-chlamydial antibodies in serum by contacting a serum sample from a subject with at least one of the polypeptide fragments of the invention and using well-known methods for visualising the reaction between the antibody and antigen.

15 In diagnostics, an antibody, a nucleic acid fragment and/or a polypeptide of the invention can be used either alone, or as a constituent in a composition. Such compositions are known in the art, and comprise compositions in which the antibody, the nucleic acid fragment or the polypeptide of the invention is coupled, preferably covalently, to at least one other molecule, e.g. a label (e.g. radioactive or fluorescent) or a carrier molecule.

20

The present invention discloses antigenic components of *C. trachomatis* which have:

- 1) the capacity to stimulate T cells from patients with a urogenital *Chlamydia* infection to secrete INF γ , or
- 2) the capacity to stimulate T cells from patients with a urogenital *Chlamydia* infection to secrete cytokines which inhibit *Chlamydia* growth in vitro, or
- 25 3) is recognized by serum IgG, and / or IgM, and / or IgA, antibodies from patients with a urogenital chlamydia infection, or
- 4) is recognized by T cells and / or antibodies from mice experimentally infected with *Chlamydia muridarum* and / or *C. trachomatis*, or
- 30 5) is able by administration to induce an immune response in mice which recognize the *C. trachomatis* bacterial antigen, or
- 6) is able by vaccination to provide at least partial immunity against an experimental

challenge infection with *Chlamydia muridarum* and / or *C. trachomatis*.

Firstly, in order to identify the molecular targets of protective T cells among proteins from
5 *C. trachomatis*, a protein lysate of *C. trachomatis* serovar D (strain UW-3/Cx, ATCC No:
VR-885) was fractionated by the multi-elution technique (Andersen and Heron 1993). This
technique separates proteins in a complex protein mixture according to their molecular
weight into narrow fractions which are then used to stimulate Peripheral Blood
Mononuclear Cells (PBMCs) *in vitro*. After several days of incubation the release of INF γ
10 is monitored by ELISA (Fig 1). The responses of Chlamydia patients were compared to the
responses of normal blood donors with no previous diagnosis of Chlamydia infection. This
comparison allows identification of *C. trachomatis* proteins which have the capacity to
trigger effector T cells to release INF γ during the first phases of the human infection.
Using this approach it was demonstrated that the targets for these protective T cells are
15 proteins or fragments of proteins with apparent molecular weights of 5-12, 16-20, 25-35
and 58-74 kDa (Fig's 2 and 3). The precise identity of bacterial proteins within each
stimulatory region was determined by mass spectrometry.

To further identify and characterise the stimulating antigens, each specific *C. trachomatis*
20 antigens may be a) purified antigens from *C. trachomatis* extracts as exemplified in
Example 1, b) antigens produced and purified from *E. coli* as exemplified in Example 1, c)
overlapping synthetic peptides as exemplified in Example 1, or d) transduction of target
patient PBMC directly with recombinant Adenovirus constructs as exemplified by
Example 5. This method enabled the identification of single antigens and peptides derived
25 thereof within each stimulatory region with exceedingly stimulating capacity measured by
the release of INF γ as exemplified in Figure 4.

Secondly, a directed expression-library was constructed by amplifying full-length *C.*
trachomatis genes by polymerase chain reaction (PCR) using gene-specific
30 oligonucleotides containing a Kozak sequence in the 5'-primer and a stop codon in the 3'-
primer. Genomic DNA from *C. trachomatis* serovar D was used as template for the PCR
reactions and a newly developed UNIX program was used for automated primer design

including primer position within the gene of interest and Tm. Amplicons were first inserted by recombination into the Gateway "entry vector" (Invitrogen) and then transferred by recombination into the pDEST17 expression vector (Invitrogen), which contains a His₆-tag and the same recombination sequences as the entry vector. Individual clones were screened for the expression of *C. trachomatis* antigens by the colony blot method (French *maul and maul* 1986) using a pool of human serum samples with high levels of *C. trachomatis* specific IgG, IgM, or IgA antibodies. The nitrocellulose filters used for the colony lift had been pre-soaked in 1% arabinose solution in order to induce transcription originating for the plasmid encoded promoter prior to cell lysis. Positive clones which bind to serum IgG, IgM, or IgA antibodies from chlamydia patients were selected for further analysis by western blotting using the same pool of serum samples as used for the initial screening. This method led to the identification of clones encoding immunoreactive *C. trachomatis* proteins of vaccine and diagnostic relevance..

15 Thirdly, a genomic expression library was constructed in *E. coli* phage lambda gt11 (λ gt11). High-molecular-weight chromosomal DNA of *C. trachomatis* serovar D was extracted from elementary bodies in a lysis buffer containing SDS (1%) and Proteinase K (100ug/ml) followed by phenol extraction and ethanol precipitation. DNA was partially degraded by sonication and DNA fragments of 0,2 - 0,8kb in size were ligated into λ gt11.

20 The ligation mixture was packaged *in vitro* and the recombinant phages were plated on *E. coli* Y1090r- yielding a genomic expression library containing approximately 3.4×10^5 primary lambda phages. This primary library was amplified to resulting in a genome random expression library with 6.7×10^9 PFU/ml. In a first experiment, this library was screened by a plaque-lift method using the same pool of human serum samples as used above for the screening of the full-length expression library. Eighty-eight immuno reactive plaques binding to *C. trachomatis*-specific IgG, IgM, or IgA antibodies were identified. These plaques were pooled into eight pools (two pools of IgA reactive plaques, five pools of IgG reactive plaques and one pool of IgM reactive plaques) and rescreened with the same serum-pool (primary antibody) as used in the initial screening. Individual sero reactive phage plaques were isolated and the sequences of the DNA inserts of individually sero reactive phages were determined. This method identified a several clones encoding specific *C. trachomatis* immunoreactive peptides of vaccine and diagnostic relevance.

30

Lastly, animal models of the disease have been established in small rodent in order to identify antigens which are recognized by the murine immunesystem during an experimental *Chlamydia* infection or provides at least partial immunity against a challenge

5 infection. Different *chlamydia* species exhibit a high degree of specificity towards their natural host. Thus, *C. trachomatis* serovar D used in the different screening strategies described above is a human pathogen, which does not cause pathological changes in mice as normally associated with the human infection. On the other hand, mice can be experimentally infected with the closely related *Chlamydia muridarum* MoPn strain, and

10 several researchers have previously demonstrated induction of partial immunity against experimental MoPn infection. A genital infection model has therefore been established and validated in C57 mice. The protective efficacy of different antigens was studied in this model by evaluating 1) bacterial counts by cervical swaps, 2) pathological changes in the genital tract, and 3) cellular in vitro assays for immune reactive cells.

Table 1: Chlamydia antigens

Chlamydia antigen	Protein sequence	DNA sequence
CT043	SEQ ID NO. 1	SEQ ID NO. 2
CT511	SEQ ID NO. 3	SEQ ID NO. 4
CT521	SEQ ID NO. 5	SEQ ID NO. 6
CT616	SEQ ID NO. 7	SEQ ID NO. 8
CT803	SEQ ID NO. 9	SEQ ID NO. 10
CT067	SEQ ID NO. 11	SEQ ID NO. 12
CT679	SEQ ID NO. 13	SEQ ID NO. 14
CT583	SEQ ID NO. 15	SEQ ID NO. 16
CT603	SEQ ID NO. 17	SEQ ID NO. 18
CT026	SEQ ID NO. 19	SEQ ID NO. 20
CT093	SEQ ID NO. 21	SEQ ID NO. 22
CT357	SEQ ID NO. 23	SEQ ID NO. 24
CT659	SEQ ID NO. 25	SEQ ID NO. 26
CT111	SEQ ID NO. 27	SEQ ID NO. 28
CT509	SEQ ID NO. 29	SEQ ID NO. 30
CT587	SEQ ID NO. 31	SEQ ID NO. 32
CT023	SEQ ID NO. 33	SEQ ID NO. 34
CT025	SEQ ID NO. 35	SEQ ID NO. 36
CT078	SEQ ID NO. 37	SEQ ID NO. 38
CT082	SEQ ID NO. 39	SEQ ID NO. 40
CT118	SEQ ID NO. 41	SEQ ID NO. 42
CT174	SEQ ID NO. 43	SEQ ID NO. 44
CT003	SEQ ID NO. 45	SEQ ID NO. 46
CT005	SEQ ID NO. 47	SEQ ID NO. 48
CT027	SEQ ID NO. 49	SEQ ID NO. 50
CT032	SEQ ID NO. 51	SEQ ID NO. 52
CT008	SEQ ID NO. 53	SEQ ID NO. 54
CT016	SEQ ID NO. 55	SEQ ID NO. 56

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CT028	SEQ ID NO. 57	SEQ ID NO. 58
CT035	SEQ ID NO. 59	SEQ ID NO. 60
CT141	SEQ ID NO. 61	SEQ ID NO. 62
CT643	SEQ ID NO. 63	SEQ ID NO. 64
CT414	SEQ ID NO. 65	SEQ ID NO. 66
CT874	SEQ ID NO. 67	SEQ ID NO. 68
CT456	SEQ ID NO. 69	SEQ ID NO. 70
CT681	SEQ ID NO. 71	SEQ ID NO. 72
CT123	SEQ ID NO. 73	SEQ ID NO. 74
CT125	SEQ ID NO. 75	SEQ ID NO. 76
CT126	SEQ ID NO. 77	SEQ ID NO. 78
CT133	SEQ ID NO. 79	SEQ ID NO. 80
CT150	SEQ ID NO. 81	SEQ ID NO. 82
CT175	SEQ ID NO. 83	SEQ ID NO. 84
CT376	SEQ ID NO. 85	SEQ ID NO. 86
CT083	SEQ ID NO. 87	SEQ ID NO. 88
CT089	SEQ ID NO. 89	SEQ ID NO. 90
CT155	SEQ ID NO. 91	SEQ ID NO. 92
CT168	SEQ ID NO. 93	SEQ ID NO. 94
CT184	SEQ ID NO. 95	SEQ ID NO. 96
CT124	SEQ ID NO. 97	SEQ ID NO. 98
CT336	SEQ ID NO. 99	SEQ ID NO. 100
CT342	SEQ ID NO. 101	SEQ ID NO. 102
CT842	SEQ ID NO. 103	SEQ ID NO. 104
CT323	SEQ ID NO. 105	SEQ ID NO. 106
CT080	SEQ ID NO. 107	SEQ ID NO. 108
CT084	SEQ ID NO. 109	SEQ ID NO. 110
CT110	SEQ ID NO. 111	SEQ ID NO. 112
CT119	SEQ ID NO. 113	SEQ ID NO. 114
CT541	SEQ ID NO. 115	SEQ ID NO. 116
CT443	SEQ ID NO. 117	SEQ ID NO. 118

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CT795	SEQ ID NO. 119	SEQ ID NO. 120
CT396	SEQ ID NO. 121	SEQ ID NO. 122
CT283	SEQ ID NO. 123	SEQ ID NO. 124
CT051	SEQ ID NO. 125	SEQ ID NO. 126
CT002	SEQ ID NO. 185	SEQ ID NO. 186
CT009	SEQ ID NO. 187	SEQ ID NO. 188
CT015	SEQ ID NO. 189	SEQ ID NO. 190
CT030	SEQ ID NO. 191	SEQ ID NO. 192
CT048	SEQ ID NO. 193	SEQ ID NO. 194
CT061	SEQ ID NO. 195	SEQ ID NO. 196
CT063	SEQ ID NO. 197	SEQ ID NO. 198
CT068	SEQ ID NO. 199	SEQ ID NO. 200
CT071	SEQ ID NO. 201	SEQ ID NO. 202
CT115	SEQ ID NO. 203	SEQ ID NO. 204
CT678	SEQ ID NO. 205	SEQ ID NO. 206
CT561	SEQ ID NO. 207	SEQ ID NO. 208
CT538	SEQ ID NO. 209	SEQ ID NO. 210
CT582	SEQ ID NO. 211	SEQ ID NO. 212
CT875	SEQ ID NO. 213	SEQ ID NO. 214
CT322	SEQ ID NO. 215	SEQ ID NO. 216
CT112	SEQ ID NO. 217	SEQ ID NO. 218
CT315	SEQ ID NO. 219	SEQ ID NO. 220
CT610	SEQ ID NO. 221	SEQ ID NO. 222
CT147	SEQ ID NO. 223	SEQ ID NO. 224
CT228	SEQ ID NO. 225	SEQ ID NO. 226
CT232	SEQ ID NO. 227	SEQ ID NO. 228
CT614	SEQ ID NO. 229	SEQ ID NO. 230
CT098	SEQ ID NO. 231	SEQ ID NO. 232
CT265	SEQ ID NO. 233	SEQ ID NO. 234
CT375	SEQ ID NO. 235	SEQ ID NO. 236
CT004	SEQ ID NO. 237	SEQ ID NO. 238

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CT038	SEQ ID NO. 239	SEQ ID NO. 240
CT040	SEQ ID NO. 241	SEQ ID NO. 242
CT052	SEQ ID NO. 243	SEQ ID NO. 244
CT053	SEQ ID NO. 245	SEQ ID NO. 246
CT201	SEQ ID NO. 247	SEQ ID NO. 248
CT245	SEQ ID NO. 249	SEQ ID NO. 250
CT246	SEQ ID NO. 251	SEQ ID NO. 252
CT405	SEQ ID NO. 253	SEQ ID NO. 254
CT420	SEQ ID NO. 255	SEQ ID NO. 256
CT426	SEQ ID NO. 257	SEQ ID NO. 258
CT507	SEQ ID NO. 259	SEQ ID NO. 260
CT512	SEQ ID NO. 261	SEQ ID NO. 262
CT513	SEQ ID NO. 263	SEQ ID NO. 264
CT514	SEQ ID NO. 265	SEQ ID NO. 266
CT516	SEQ ID NO. 267	SEQ ID NO. 268
CT316	SEQ ID NO. 269	SEQ ID NO. 270
CT439	SEQ ID NO. 271	SEQ ID NO. 272
CT492	SEQ ID NO. 273	SEQ ID NO. 274
CT520	SEQ ID NO. 275	SEQ ID NO. 276
CT523	SEQ ID NO. 277	SEQ ID NO. 278
CT526	SEQ ID NO. 279	SEQ ID NO. 280
CT611	SEQ ID NO. 281	SEQ ID NO. 282
CT613	SEQ ID NO. 283	SEQ ID NO. 284
CT626	SEQ ID NO. 285	SEQ ID NO. 286
CT630	SEQ ID NO. 287	SEQ ID NO. 288
CT647	SEQ ID NO. 289	SEQ ID NO. 290
CT649	SEQ ID NO. 291	SEQ ID NO. 292
CT725	SEQ ID NO. 293	SEQ ID NO. 294
CT734	SEQ ID NO. 295	SEQ ID NO. 296
CT779	SEQ ID NO. 297	SEQ ID NO. 298
CT801	SEQ ID NO. 299	SEQ ID NO. 300

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CT833	SEQ ID NO. 301	SEQ ID NO. 302
CT835	SEQ ID NO. 303	SEQ ID NO. 304
CT836	SEQ ID NO. 305	SEQ ID NO. 306
CT845	SEQ ID NO. 307	SEQ ID NO. 308

Table 2: Chlamydia antigenic fragments

Peptide fragment	Amino acid sequence	DNA sequence
CT541-PF1 (aa pos. 111-243)	SEQ ID NO. 127	SEQ ID NO. 128
CT443-PF1 (aa pos. 214-291)	SEQ ID NO. 129	SEQ ID NO. 130
CT795-PF1 (aa pos. 1-163)	SEQ ID NO. 131	SEQ ID NO. 132
CT396-PF1 (aa pos. 170-318)	SEQ ID NO. 133	SEQ ID NO. 134
CT842-PF1 (aa pos.433-515)	SEQ ID NO. 135	SEQ ID NO. 136
CT283-PF1 (aa pos. 477-577)	SEQ ID NO. 137	SEQ ID NO. 138
CT874-PF1 (aa pos. 330-426)	SEQ ID NO. 139	SEQ ID NO. 140
CT051-PF1 (aa pos. 38-177)	SEQ ID NO. 141	SEQ ID NO. 142
CT141-PF1 (aa pos. 17-126)	SEQ ID NO. 143	SEQ ID NO. 144
CT643-PF1 (aa pos. 769-841)	SEQ ID NO. 145	SEQ ID NO. 146
CT681-PF1 (aa pos. 156-391)	SEQ ID NO. 147	SEQ ID NO. 148
CT681-PF2 (aa pos. 199-329)	SEQ ID NO. 149	SEQ ID NO. 150
CT681-PF3 (aa pos. 294-349)	SEQ ID NO. 151	SEQ ID NO. 152
CT414-PF1 (aa pos. 605-722)	SEQ ID NO. 153	SEQ ID NO. 154
CT414-PF2 (aa pos. 463-530)	SEQ ID NO. 155	SEQ ID NO. 156
CT456-PF1 (aa pos. 695-840)	SEQ ID NO. 157	SEQ ID NO. 158
CT456-PF2 (aa pos. 137-229)	SEQ ID NO. 159	SEQ ID NO. 160
CT456-PF3 (aa pos. 243-321)	SEQ ID NO. 161	SEQ ID NO. 162
CT456-PF4 (aa pos. 209-291)	SEQ ID NO. 163	SEQ ID NO. 164
CT456-PF5 (aa pos. 175-279)	SEQ ID NO. 165	SEQ ID NO. 166
CT456-PF6 (aa pos. 567-730)	SEQ ID NO. 167	SEQ ID NO. 168
CT456-PF7 (aa pos. 210-540)	SEQ ID NO. 169	SEQ ID NO. 170
CT456-PF8 (aa pos. 190-279)	SEQ ID NO. 171	SEQ ID NO. 172
CT521-PF1 (aa pos. 14-36)	SEQ ID NO. 173	SEQ ID NO. 174
CT521-PF2 (aa pos. 40-62)	SEQ ID NO. 175	SEQ ID NO. 176
CT521-PF3 (aa pos. 52-75)	SEQ ID NO. 177	SEQ ID NO. 178
CT521-PF4 (aa pos. 66-88)	SEQ ID NO. 179	SEQ ID NO. 180
CT521-PF5 (aa pos. 116-138)	SEQ ID NO. 181	SEQ ID NO. 182
CT504-PF1 (reverse)	SEQ ID NO. 183	SEQ ID NO. 184

Figure legends

Figure 1

- 5 Cellular reactivity to a *C. trachomatis* serovar D lysate. IFN γ responses of PBMC's isolated from 6 control donors and 15 patients. PBMC 's were stimulated with 5 μ g/ml of a *C. Trachomatis* lysate and IFN γ release was determined 5 days later in the supernatants.

Figure 2

- 10 Protein fractions of *C. trachomatis* serovar D. A lysate from the bacteria was separated into narrow-molecular fractions by the multielution technique. The fractions were analysed by SDS- PAGE and silver staining. The migration of molecular weight markers is shown at the right (lane 1) in kilodaltons. The lysate is shown in the third and last lanes.

15 Figure 3

Human T cell recognition of *C. trachomatis* serovar D protein fractions. PBMC's isolated from 8 Chlamydia patients (responding to the whole lysate > 1000 pg/ml) and 6 control donors were stimulated with 2 μ g/ml of the individual fractions. The release of IFN γ were measured in the supernatants 5 days later. Short line indicates the mean IFN γ release.

20

Figure 4

T cell responses to recombinant proteins in 4 patients and 3 controls. PBMC's were stimulated with 5 μ g/ml of rCT521, rCT511, rCT616, r CT043 and rCT803.

Values shown means of IFN γ for triplicate cultures.

25

Figure 5

The recognition of rCT521 in 41 chlamydia patients (all responding to a *C. trachomatis* serovar D lysate with more than 1500 pg/ml of IFN γ , and 11 control donors responding with less than 1500 pg/ml of IFN γ to the lysate. PBMC's were stimulated with rCT521 (5 μ g/ml) and a pool of overlapping CT521 peptides (10ug/ml each) and the level of IFN γ were measured in the supernatants. C: Control wells without antigen. Short Lines indicate the mean IFN γ (pg/ml).

30

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34

Figure 6

INF γ release stimulated with CT521 peptides (10ug/ml). Short lines indicate the mean INF γ release for each peptide. Cut off is set to 200pg/ml INF γ (line).

5 Figure 7

INF γ release by PBMC transduced with different recombinant Adenovirus encoding *C. trachomatis* antigens. Patient PBMC were transduced with indicated Adenovirus at a multiplicity of infection of 1, and INF γ release was determined at day two. AdVaMock indicates activity of a transduced Adenovirus without insert.

10

Figure 8

Inclusion Forming Units at PID7 and PID14

Figure 9

15 Hydrosalpinx scores at PID49

Figure 10

T cell responses to *C. Trachomatis* proteins (Fig 10a-10i). The proteins were tested in 10 patients (●) and 5 controls (□). C, cell cultures without antigen. Values shown are median and 75 and 25% percentiles.

20

Figure 11

T cell responses to *C. Trachomatis* proteins where 5 or more patients responds with a level of IFN- γ above all controls. The proteins were tested in 10 patients (●) and 5 controls (□). C, cell cultures without antigen. Values shown are median and 75 and 25% percentiles.

25

Figure 12

Antigen specific responses by blood lymphocytes 1 week after the last immunization. The IFN- γ response were measured in cell cultures pooled from 10 animals. Each bar represents the means of triplicate values +/- standard deviation.

30

Figure 13

Inclusion forming units 7, 14 and 21 days post infection in C3H/HeN mice. The values are shown as log₁₀ IFU/ml. All values represents the mean of 10 animals +/- Standard error of the mean.

5

Fig 14.

Serum reactivity against immunogen measured by ELISA measured as dilution at OD=1.0. Each point represents a mean of 4 animals +/- standard error of the mean.

10

Fig 15:

Specific serum reactivity against whole elementary bodies lysates from either *Chlamydia muridarum* (MoPn EB's) or *Chlamydia trachomatis* (Seroovar D EB's). Positives are marked by a red dot. Positives are bands with size in agreement with theoretical size.

15

Fig16

Antigen specific responses by splenocytes 3 weeks after the last immunization. The IFN- γ response were measured in cell cultures from 4 individual animals. Each bar represents the means of triplicate values +/- standard deviation.

20

Examples

Example 1: Identification of human T cell antigens of *C. trachomatis* serovar D

Introduction

- 5 We have analysed the human T cell responses to *C. trachomatis* proteins using narrow molecular weight fractions derived from complex protein mixtures separated by SDS-PAGE followed by electroelution. This technique enable direct analysis of the immune response and making comparison of stimulatory protein fractions possible. This has led to the identification of a number of stimulatory protein fractions and identification of T cell
- 10 targets. Further evaluation of these T cell targets have been done using recombinant technologies and overlapping peptides spanning the entire sequence of the protein.

Materials and methods

Microorganism and cultivation

- 15 *C. Trachomatis* serovar D (strain UW-3/Cx) was propagated in Hela 229 cells (ATCC, Rockville, MD, USA). The cells were cultivated in passage medium RPMI 1640 (Gibco BRL, Grand Island, NY, USA) containing 5% fetal calf serum (Gibco BRL; heat inactivated), 1% v/v Hepes, 1% v/v L-glutamine, 1% v/v pyrovate and 10 µg/ml gentamycine.
- 20 Semiconfluent monolayers of Hela 229 cells in 175 cm² flasks were pre-treated for 15 minutes at RT with DEAE-dextran (45 µg/ml in HBSS) and infected with one inclusion forming unit per cell of *C. trachomatis* serovar D in 3 ml HBSS. The flasks were incubated on a plate rocker for 2 h at 37 °C. After 2 h 50 ml passage medium RPMI 1640 supplemented with 5% glucose and 1 µg/ml cycloheximid were added pr. flask and the
- 25 cells were further incubated for 72 h in an athmosphere of 5% CO₂ in humidified air.

Harvesting of *C. trachomatis*

- Chlamydiae were harvested 72 h post infection. The cells were dislodged from the flasks with a cell scraper and centrifuged 30 minutes at 35,000g and 4°C. The pellets were
- 30 resuspended in 5 ml HBSS per flask, sonicated on ice and centrifuged at 500g and 4°C for 15 minutes. The supernatant was collected and saved on ice and the pellet was resuspended to same volume as before and sonication and centrifugation were repeated. The two supernatants were pooled and centrifuged 30 minutes at 30000g and 4°C and the pellet

resuspended with a needle and syringe in a SPG buffer (3 ml/T175). After a brief sonication the suspension was gently layered over a 30% Diatrizoate solution (50g Meglumine diatrizoate, 7.7g Sodium diatrizoate in 76ml H₂O) and centrifuged at 40,000g for 30 min. After centrifugation the pellet were resuspended in SPG buffer and stored at -70 °C.

Preparation of *C. trachomatis* lysate for fractionation

A quantity of 6-8 mg of *C. trachomatis* was centrifuged 30000g for 30 minutes and the pellet was resuspended 1:1 in WFI and samplebuffer/DTT and boiled for 5 minutes. After 2 x 12 sec. of sonication the suspension was centrifuged 30000g for 30 minutes. The supernatant was stored at -70 °C until use.

Fractionation of *C. trachomatis* lysate

C. trachomatis lysate was fractionated as described by Andersen and Heron (1993). Briefly, *C. trachomatis* lysate in a quantity of around 6-8 mg of protein was separated by SDS-page (10 to 20% gel) overnight (11-cm-wide center well, 0.75-mm gel). Gels preequilibrated in elution buffer (ammonia Caps buffer pH 10.2) were transferred to a Multi-Eluter and electroeluted for 20 min. The protein fractions were aspirated and analysed by separation on SDS 10-20% polyacrylamide gels followed by silver staining (Blum and Gross 1987). The protein concentration in the fractions was estimated by the Micro BCA method (Pierce, Oud-beijerland, The Netherlands). 0.5 ml of all fractions were stabilized by 0.5 % human AB serum and kept frozen at -70 °C until use. The rest was stored at -70°C without serum in order to be used for mass spectrometry analysis

Mass spectrometry analysis

Samples for peptide mass mapping were cut out of a silver stained SDS-PAGE gel. The band was washed, dried, reduced and alkylated with iodoacetamide before being digested overnight by modified trypsin essentially as described by Shevchenko et al, 1998.

Donors

Patients diagnosed with Chlamydia at Bispebjerg hospital, Denmark were asked to participate in the study and to give a blood sample before initiation of antibiotic therapy. Control subjects with no records of Chlamydia infections were also asked to participated in

the study. Samples from individual patients were annotated with a unique identifiable annotation by assigning a running number, either M or K for male or female, and optionally A, B, C... for 1st, 2nd, 3rd,... sample collected from the particular patient. In all cases, the A sample were collected before any treatment was initiated. For example,

5 12MB denotes the second sample taken from the male patient number 12. Control samples were annotated KK-xx.

Lymphocyte preparation and cell culture

Peripheral blood mononuclear cells (PBMC's) were separated from whole blood by lymphoprep (Nycomed A/S, Oslo, Norway) density gradient centrifugation and frozen in liquid nitrogen until use. PBMC were thawed and resuspended in RPMI 1640, supplemented with 1% penicillin/streptomycin, 1% nonessential amino acids, 1% glutamine (Gibco), 1% pyroval, 1% heepes and 10% human AB Serum (local blood bank, Rigshospitalet, Copenhagen). The viability and number of cells were determined by

15 Nigrosin staining. The cells were cultured in triplicates in round-bottom microtiter plates (Nunc, Roskilde, Denmark) at 1.25×10^5 cells/well in a total volume of 100 μ l. On the basis of initial dose-response studies, antigens were added in the following concentrations: SvD lysate: 2 μ g/ml, SvD fractions 2 μ g/ml, rCT521 5 μ g/ml, CT521 overlapping peptides 10 μ g/ml. Phytohemagglutinin (PHA, 2 μ g/ml) was used as a positive control and cell

20 cultures without antigen were included as a negative control. After 5 days of incubation at 37 °C in humidified air (5% CO₂ and 95% air), the supernatants were harvested.

IFN γ assay

The amount of IFN γ in the supernatants were determined by ELISA with commercially available antibodies (Endogen) and used according to the manufacturer's instructions.

25 Recombinant IFN γ was used as a standard (Endogen).

Overlapping peptides

10 synthetic 22-23 mer peptides (9-12 aa overlap) covering the complete primary sequence of CT521 were synthesized by solid phase methods (Schafer-N).

30

Production of *C. trachomatis* antigens in *E. coli*.

The CT genes encoding antigens identified by mass-spectrometry were cloned *in frame*

with the NH₂-terminal (His)₆ sequence of the pDEST17 vector according to the Gateway Cloning Technology Manual (Invitrogen). For production of the recombinant *C. trachomatis* antigens, the plasmid vectors were cloned in the BL21-AI *E. coli* strain (Invitrogen) facilitating high-level recombinant protein production in the presence of
5 arabinose.

Mini-scale purification of recombinant *C. trachomatis* antigens.

Bacterial cell pellets were suspended in 10mM Imidazole, 20mM NaH₂PO₄, 500mM NaCl, 8M Urea, subjected to cell disruption by BeadBeater according to manufacturer's
10 instructions (BioSpec Products, Inc.), following incubation with gentle shaking at room temperature for 1h. The cleared supernatant was applied on a HisTrap column (Pharmacia Biotech), washed and eluted with 0.5M Imidazol, 20mM NaH₂PO₄, 500mM NaCl, 8M Urea. The eluted sample was separated by electrophoresis on a preparative SDS-PAGE. The recombinant polypeptide of interest was identified by Coomassie-Blue stain, cut out
15 and electro eluted from the gel piece using the Model 422 Electro-Eluter according to Instruction manual (BioRad). The electro eluted recombinant antigen was precipitated in 80%-95% Acetone (Aldrich HPLC grade), washed in 95% Ethanol, and resuspended in a minimal volumen of 10mM Imidazole, 20mM NaH₂PO₄, 500mM NaCl, 8M Urea. The sample was finally dialysed to 50mM Tris pH 7.5; 150mM NaCl, 40% glycerol and stored
20 at -20°C.

Results:

T cell response to a Chlamydia lysate

Chlamydia patients were screened for their T cell recognition of a *C. Trachomatis* serovar
25 D lysate harvested 72 h post infection of Hela cells. The lysate represents a mixture of all the components of the bacteria and cover the whole antigen repertoire of the bacteria. This preparation was used to stimulate PBMCs from 15 Chlamydia patients and 6 control donors (Fig. 1). The response to the lysate was associated with a pronounced level of IFN γ (> 1000 pg/ml) in 8 out of 15 patients. Only one control donor responded to the lysate with
30 more than 1000 pg/ml of IFN γ .

Chlamydia patients recognize multiple antigens

The specificity of the T cell response was investigated by stimulating PBMCs with protein

fractions obtained by the multielution technique. The technique was used on the lysate and resulted in narrow fractions with a minimal overlap between neighbouring fractions (Fig. 2). The numbers of polypeptides in each fraction are estimated to be 10 to 30. Such a panel of fractions was used to screen the antigen recognition patterns of the 8 patients responding to the whole lysate and the 6 control donors (Fig. 3). The cellular response to the fractions showed that the response was directed to multiple antigens. Peak production of IFN γ was however observed in the molecular mass regions 5-12, 16-20, 25-35 and 58-74 KDa.

10 Recognition of recombinant proteins by Chlamydia patients

An SDS page was run with fraction 7 and the neighbouring fractions 6 and 8 covering the molecular mass region 16-20 (Fig. 2), the gel was silverstained and the areas containing the fractions were cut out of the gel, placed in Milli Q water and sent to mass-spectrometry for protein identification. Six hits were identified: CT521, CT043, CT511, CT616, CT315 and CT803. Further more fraction 10, 11, 12, 13, 14 and 15 covering the molecular mass region 25-35 were sent to mass-spectrometry. Ten hits were identified: CT603, CT678, CT561, CT610, CT538, CT582, CT583, CT679, CT067, CT681. Fraction 22 covering the molecular mass region 58-74 was sent to mass-spectrometry. Three hits were identified CT875, CT110, CT112. Finally a fraction 18 was sent to mass spectrometry and 2 hits were identified: CT587 and CT322.

The recombinant proteins, rCT043, rCT511, rCT521, rCT616, rCT803, were purified from *E. coli* and the immunological activities of the 5 *C. trachomatis* proteins were investigated in 4 patients 1KA, 15KA, 7KA and 12KA (Fig. 4). rCT521 was the most promising antigen out of the 4 tested. Three out of 4 patients (1KA, 7KA and 15KA) responded strongly (> 1000 pg/m) to rCT521 compared to the control donors. rCT803, rCT511 and rCT616 induced high levels of IFN γ in two (1KA, 7KA) out of four patients whereas rCT043 induced low levels of IFN γ in all patients. The recombinant proteins CT043, CT511, CT603, CT561, CT610, CT583, CT679, CT067, CT681 CT875, CT110, CT112 CT587 and CT322 were produced in E-coli and tested for T cell recognition in 10 patient and 5 controls (Example 8)

CT521 recognition by Chlamydia patients

The recognition of CT521 by Chlamydia infected patients were tested in a larger panel of donors. A total of 41 chlamydia patients all responding to a Chlamydia lysate with more
5 than 1500 pg/ml of IFN γ were tested for recognition of CT521. In addition 11 control donors responding with less than 1500 pg/ml of IFN γ to the lysate were included (Fig. 5). Patients could be divided into CT521 positive and CT521 negative on the basis of IFN γ responses exceeding 500 pg/ml. 34 out of the 41 patients were CT521 positive (82.9 %) whereas only two out of 11 controls responded to CT521 (18.2 %). These results
10 demonstrate that CT521 is frequently recognized by Chlamydia patients responding to the whole Chlamydia lysate.

Fine specificity of the T cell response to CT521 mapped by synthetic peptides

15 The fine specificity of the T cell responses to CT521 was mapped by screening a panel of overlapping peptides covering the complete CT521 sequence. The peptides were synthesized as 22-23 mers with 9-12 amino acid overlap and were used to stimulate PBMC from 41 Chlamydia patients and 11 controls (Fig. 6). Even though the response was highly heterogeneous certain hierarchy existed with certain regions being strong targets for the
20 response. Epitopes present in the N-terminal part of the protein (aa14 to aa36), the central part (aa40 to aa88), and in the C-terminal part (aa116 to aa138) of the protein were more strongly or more frequently recognized than the others.

25 Example 2: Directed library strategy (screening for antibody targets)

Introduction

A High Throughput approach was taken to test for serum reactive antigens in the *C. trachomatis* serovar D genome. A full length library was constructed of the first 200 Open
30 Reading Frames (ORFs). This library was designed to express the antigens recombinantly in *Escherichia coli*. For screening of this library, we used a pool of serum from 5 high responding patients which were selected based on their reactivity towards a whole *C. trachomatis* Elementary Body (EB) extract by Western blot analysis.

Materials and methods

Construction of full length library

The genome of *C. trachomatis* serovar D is publicly available and the primary annotation
5 was used as defined by Stephens et. al. (Stephens, Kalman et al. 1998). Genes Ct001 to
Ct200 was selected for cloning. 5' and 3' primers for amplification of the specific genes
was designed by a "in house" software. The full length sequences of the 200 specific *C.*
trachomatis genes were cloned into the Entry Vector, pDONR 201 (Invitrogen), which
enable to clone the genes of interest into different destination vectors of the Gateway
10 cloning system (Invitrogen). The pDEST17 destination vector was used for expression of
the recombinant *C. trachomatis* protein in *E. coli* with a 6XHistidine affinity tag. The
bacterial host was BL21-AITM for production of the recombinant *C. trachomatis* proteins by
induction with arabinose.

15 Expression

2*96 Deep Well plates containing 1 ml cultures of were grown over night at 37°C. The
culture was diluted to OD₆₀₀ = 0.1 and incubated at 37°C with shaking (180 rpm) until
OD₆₀₀ = 0.5 was reached then the culture was induced by adding L-arabinose to a final
concentration of 0.2%. After 4 hours of induction the cultures were put on ice and the
20 bacterial pellet was collected by centrifugation (3.000g/20min.). Pellets were kept in the
fridge until results from the colony blot was obtained.

Patient serum

Serum from five positive *C. trachomatis* patients, 3KA, 11KA, 12KA, 13KA, and 17KA,
25 was selected for preparing a patient serum pool to be used in the library screening. These
patient sera were selected by their specific and high reactivity against *C. trachomatis*
serovar D elementary body extract in Western blot analyses using alkaline phosphatase
conjugated rabbit anti-human -IgA, -IgG, and -IgM, respectively, as secondary detection
marker (DakoCytomation, Denmark).
30 The patient serum pool (diluted 10 times) was pre-treated with total *E. coli* protein extract
at 2mg/ml for 3h at room temperature. The working patient pool serum was 1:200 in
10mM Tris-HCl, pH 8, 150mM NaCl, 0.05% Tween20 (TBST).

Colony blot

Screening of the full length *E. coli* expression library was basically performed according to French et al. (1986). Bacterial cultures (1ml) encoding the Ct001-Ct200 and selected
 5 genes throughout the genome were grown over night at 37°C in two 96 Deep Well plates. Using a (6 x 8) gripper tool, the bacterial cultures were transferred to Petri dishes containing LB-agar (containing 100ug/ml ampicillin). The colonies were left over night at 30°C. The colonies were replicated onto a nitrocellulose membrane presoaked in 1% L-arabinose and transferred to new LB-agar plates (with 100ug/ml ampicillin and 0.2% L-arabinose)
 10 with the colony side down. The plates were incubated at 37°C for 4 hours and finally the membranes were transferred to an empty Petri dish with colony side up for 15min over a filter paper presoaked in chloroform, thereby exposing the bacteria to chloroform vapor. The membranes were incubated over night in lysis buffer containing lysozyme and DNase. After repeated washing steps the membranes were incubated with
 15 primary antibody (working patient pool serum) for 2 hours at room temperature. The membranes were washed repeatedly (4 times with excess 1 x TBST) before incubating in secondary antibody for 1h. The second antibody was either:

- A. Rabbit anti human IgG (DO336) DakoCytomation
- B. Rabbit anti human IgA (DO338) DakoCytomation
- 20 C. Rabbit anti human IgM (DO337) DakoCytomation
- or
- D. A pool of Rabbit anti human IgG (DO336) and Rabbit anti human IgA (DO338)

25 All conjugated to alkaline phosphatase.

After a second washing in 1 x TBST, the membranes were developed by BCIP/NBT substrate (Sigma Fast).

Positive clones were selected in all categories (IgG, IgA and IgM).

30

Western blot of clones positive in colony blot

Bacterial pellets from the 1 ml cultures were resuspended in 200µl SDS-PAGE sample buffer and heated to 95°C for 5 min, electrophoresed by SDS-PAGE and transferred to

nitrocellulose by standard Western blotting method. The membranes were incubated with the same patient serum pool and a pool of the secondary antibodies (A-C) as described above. As a control for protein induction a replicate membrane was incubated with anti Penta-His antibody and processed according to the manufacturer's instructions (Qiagen).

- 5 Two colonies that did not react in the colony blot were included as controls.

Results:

The identity of the CT antigens recognized by patient serum in the bacterial colony screening approach is:

10

Antigen	IgG	IgA	IgM	Western Blot
Ct080		+		++
Ct084			+++	+
Ct089	++			++
Ct110	+++	++		+
Ct115	++	++		+
Ct118	++			+
Ct119	++			+++
Ct125		+	++	++
Ct147		+		++
Ct155	+++	+++		-
Ct168		+		+
Ct174		++		++
Ct184		+		++
Ct228		+		++
Ct232	+			+
Ct614		+		+
Ct795	+++	+++		+++

where +, ++, +++, and -, indicate relative "visual intensity of reactivity" when analysed on colony blot or by Western blot.

Example 3: Random library strategy

Introduction

- 5 In order to screen for serum reactive antigen in the *C. trachomatis* serovar D genome, a random expression library was constructed in the expression vector λ gt11. This library was designed to express randomly *C. trachomatis* peptide fragments of 100-400 amino acid residues fused *in frame* with the β -galactosidase. The library was screened with serum
10 *trachomatis* Elementary Body (EB) extract by Western blot analysis.

Materials and methods

Isolation of high molecular weight *C. trachomatis* serovar D genomic DNA.

- A crude *C. trachomatis* serovar D elementary body preparation containing $\sim 8.6 \times 10^9$ IFU
15 (infectious units) was further purified by 44-54% Diatrizoate solution step density gradient ultracentrifugation at 40.000xg for 60min. The elementary bodies banded at the 54% interface were collected, diluted in 10 volumes SPG buffer (250mM Sucrose; 10mM Na_2HPO_4 ; 5mM L-Glutamic acid), and precipitated by centrifugation at 30.000xg for 30min. The elementary body pellet was resuspended in 5ml TENS buffer (50mM Tris pH
20 9; 100mM EDTA; 200mM NaCl; 1% SDS) and incubated with 100ug/ml Proteinase K at 37°C for 60min. The sample was diluted once in TENS buffer and the nucleic acids were purified by phenol/chloroform extraction and ethanol precipitation (Maniatis et al., 1987). RNA was removed by treatment with 25U/ml RNaseT1 and RNaseA cocktail (Stratagene) at 37°C for 60min followed by another phenol/chloroform extraction and ethanol
25 precipitation. The *C. trachomatis* serovar D genomic DNA preparation was resuspended in TE at 0.4ug/ul and an aliquot was tested by agarose gel electrophoresis and shown to contain high molecular weight DNA $\gg 50\text{kb}$.

Construction of whole-*C. trachomatis*-genome random expression library.

- 30 The generation of random *C. trachomatis* genomic DNA fragments by sonication was performed by placing a microcentrifuge tube containing 50ug of DNA in 175ul TM buffer (10mM Tris pH8; 10mM MgCl_2) into an ice-water bath placed in Soniprep150 sonicator

(MSE). The microtip (1/8" Ø) was placed ~2 mm below the sample surface and sonication was performed continuously for 80min at 15 microns amplitude. Under these conditions the genomic DNA was randomly fractionated to fragment size ranges of 0.05 to 1kb when analysed by agarose gel electrophoresis. During the subsequent preparation of the sonicated CT genomic DNA phenol/chloroform extraction and ethanol precipitation steps are included when appropriate. End-repair and phosphorylation of ~10ug sonicated DNA fragments were performed in a combined incubation with T4 DNA polymerase, Klenow DNA polymerase and T4 polynucleotide kinase. Furthermore, the random fragmented CT genome DNA was subjected to *EcoRI* Methylase treatment prior to linker ligation with 50-fold molar excess phosphorylated *EcoRI* linker (12-mer, BioLabs). The DNA was treated with *EcoRI* and the final DNA preparation was size fractionated on a 6% acrylamide gel and fragments of 0.2 - 0.8kb in size were eluted from the gel piece by incubation in 500ul GES buffer (0.5M NH₄Acetate; 10mM MgAcetate; 0.1mM EDTA; 0.1% SDS) at 42°C over night. The cleared supernatant was ethanol precipitated twice, and the final pellet was resuspended in 10ul TE. The DNA was ligated to *EcoRI* digested and dephosphorylated λgt11 phage vector arms (Stratagene). The ligation mix was packaged *in vitro* with Gigapack III Gold extracts according to the manufacturer's instructions (Stratagene). Recombinant phages were plated on *E. coli* Y1090r- and a total of ~340,000 primary lambda phages were generated of which ~60% were true recombinant phages as judged by the blue/white color selection assay upon plating phages in the presence of IPTG and X-gal. The primary phage expression library was amplified at densities of approximately 3 x 10⁴ PFU/ 135mm Ø plate, collected and stored in aliquots in 7% v/v DMSO at -80°C. The titer of the amplified whole-*C. trachomatis*-genome random expression library was 6.7 x 10⁹ PFU/ml.

25

Patient serum

The patient pool serum used in the screening of the random expression library was identical to the previously described in Example 2.

30 Screening the whole-*C. trachomatis*-genome random expression library

The amplified λgt11 expression library was absorbed to *E. coli* Y1090r- cells and plated at 5 x 10⁴ – 1 x 10⁵ PFU per 135mm agar plate and incubated at 42°C for 3½h. Plates are

overlaid with dried nitrocellulose membrane filters (BioTrace NT, Pall Corporation) pre-saturated with 10mM IPTG in H₂O and further incubated at 37°C for additional 3½h. The filters were transferred to TBST containing 1.5% BSA and incubated at RT for 30min, following incubation with 1:200 diluted patient pool serum at RT for 30min. Excess patient serum is removed by 3 washings in TBST for 10min each, following incubation with either alkaline phosphatase conjugated rabbit anti-human -IgA, -IgG, or -IgM at RT for 30min. After final 3 washings in TBST for 10min each, the filters were developed by BCIP/NBT substrate (Sigma Fast).

Positive immunoreactive plaque areas are collected in pools of 10 areas, titered, and re-screened at plating densities of $2.5-5 \times 10^3$ PFU per 135mm agar plate for identification of individual positive plaques.

DNA sequencing and sequence analysis.

The individual positive selected phage plaques were picked by pouncing the plaque area, suspended in 20ul H₂O, vortexed for 10sec and incubated at 37°C for 15min. The suspension was centrifuged in microfuge at maximum speed for 30sec, and 4.5ul of the cleared supernatant was used for PCR amplification using 2.5pmol each of Forward primer, 5'-ccagccatcgccatctgctgcacg-3', and λ gt11 EcoRI Reverse Primer (BioLabs) and one volume of Hot StarTaq Master Mix (Qiagen). The remaining phage suspension was diluted in 100ul SM buffer and stored as phage stock at 4°C with 25ul CHCl₃.

The PCR amplification was performed in a Gene Amp PCR System 9700 thermocycler (Applied Biosystem) at 95°C for 15min, and then 30 cycles at 95°C for 1min, 60°C for 1min, and 72°C for 1min. Four ul were tested by agarose gel electrophoresis. For sequencing the amplified DNA, the remaining 6ul of PCR reaction is diluted five-fold and purified by MicroSpin S-300 HR columns according to the manufacturer's instructions (Amersham Biosciences). The sequencing was performed by the dideoxy chain termination method (contracted by MWG-BIOTECH, Germany) using either the sequencing primer, 5'-CACCAGACCAACTGGTAATG -3', priming 28bases downstream the *EcoRI* cloning site in the *LacZ* gene, or the 5'-GCCATCGCCATCTGCTGCACG-3', priming 85 bases upstream the *EcoRI* cloning site in the *LacZ* gene. Sequences were analysed with Vector NTI Suite software package (InforMax).

Results:Identification of *C. trachomatis* sero-reactive antigens by expression library screening.

The first screening of the *C. trachomatis* λ gt11 expression library using the pooled patient
 5 serum as primary antibody identified several immunoreactive plaques areas when using
 either anti-human IgA, -IgG, or IgM as secondary detection antibodies, respectively. In
 summary, 88 positive plaques areas were picked and pooled:

No. of plaque areas picked for rescreening:

10	<u>Seroreactive Class</u>	<u># plaque hits</u>	<u># pools for screening</u>
	IgA	24	2 pools á 12 plaque areas
	IgG	50	5 pools á 10 plaque areas
	<u>IgM</u>	<u>14</u>	<u>1 pool á 14 plaque areas</u>
	Total	88	8 pools

15

The generated phage pools were rescreened using the same screening conditions as at the
 initial screening except that the plating density was much lower in order to enable
 identification of individual positive phage plaques. In summary, a total of 129 individual
 positive plaques were picked, annotated and used for direct sequence analyses and
 20 generation of phage stocks, respectively:

No. of individual picked positive plaques:

	<u>Sero reactive Class</u>	<u># Individual plaques</u>
25	IgA	41
	IgG	79
	<u>IgM</u>	<u>9</u>
	Total	129

The identity of the insert expressed as β -galactosidase fusion in the individual isolated
 30 positive phages was identified by sequencing and Blast analysis (EMBL-EBI).

The identity of CT antigens were identified by screening the random expression library.

The sequences of a total of 103 individual plaques were determined and grouped in 22 unique sequence identities (PF=peptide fragment):

- CT541-PF1 (aa pos. 111-243)
- CT443-PF1 (aa pos. 214-291)
- 5 CT795-PF1 (aa pos. 1-163)
- CT396-PF1 (aa pos. 170-318)
- CT842-PF1 (aa pos. 433-515)
- CT283-PF1 (aa pos. 477-577)
- CT874-PF1 (aa pos. 330-426)
- 10 CT051-PF1 (aa pos. 38-177)
- CT141-PF1 (aa pos. 17-126)
- CT643-PF1 (aa pos. 769-841)
- CT681-PF1 (aa pos. 156-391)
- CT681-PF2 (aa pos. 199-329)
- 15 CT681-PF3 (aa pos. 294-349)
- CT414-PF1 (aa pos. 605-722)
- CT414-PF2 (aa pos. 463-530)
- CT456-PF1 (aa pos. 695-840)
- CT456-PF2 (aa pos. 137-229)
- 20 CT456-PF3 (aa pos. 243-321)
- CT456-PF4 (aa pos. 209-291)
- CT456-PF5 (aa pos. 175-279)
- CT456-PF6 (aa pos. 567-730)
- CT456-PF7 (aa pos. 71-180)
- 25 CT456-PF8 (aa pos. 190-279)
- CT504-PF1

30 **Example 5: Generation of recombinant adenovirus encoding *C. trachomatis* antigens.**

Introduction

In order to explore an alternative delivery route of *C. trachomatis* antigens to target cells for screening for T-cell reactivity, we constructed and tested recombinant Adenovirus

encoding the antigens by direct transduction of patient PBMC.

Materials and methods.

Construction of recombinant adenovirus stocks.

5 Recombinant adenovirus encoding selected *C. trachomatis* antigens were generated essential by using the ViraPower Adenoviral Gateway Expression System (Invitrogen) introducing the CT genes in frame with an ATG initiation codon in the context of the Kozak sequence, ACCATGG, into the pAd/CMV/V5-DEST vector (Invitrogen). Stop codons were introduced just ownstream the CT gene ORF's. Viable recombinant
10 adenovirus are produced in transfected 293A cells according to the manufacturer's instructions (Invitrogen). Primary recombinant adenoviral stocks are prepared by the freeze-thaw method and stored in aliquots at -80°C. The titers measured as TCID₅₀ in 293A cells of the recombinant adenovirus stocks were determined by the Endpoint Method.

15

Results:

Preparation of Adenovirus stocks

Full length *C. trachomatis* antigens were cloned in Adenovirus for direct transduction and expression of the CT antigens in the PBMC target cell assays.

20 The following CT antigens available as Adenovirus stocks:
CT460, CT529, CT579, CT587, CT681, CT509, CT713, CT043, CT511, CT521, CT616.

T cell response to Adenoviral transduced *C. trachomatis* antigens.

The immunological activities of four Adenovirus constructs (AdVpCT043, AdVpCT511,
25 AdVpCT521 and AdVpCT616) were investigated in 9 patients and 4 controls (Figure 7). AdVpCT521 induced a strong IFN γ response (> 500 pg/ml) in 6 out of 9 patients. AdVpCT511 were recognized with levels of IFN- γ exceeding 500 pg/ml in 4 out of 9 patients whereas AdVpCT616 and AdVpCT043 only stimulated a response in 2 and 3 patients respectively. In the control group one donor responded to AdVpCT511 and
30 AdVpCT521.

Example 6: Rodent protection strategy**Introduction**

The rodent protection strategy is used to evaluate the efficacy of Chlamydia antigens. Briefly, animals immunized with antigens will be infected with a vaginal challenge of *C. muridarum*. The protective capability of the immunizing antigen will be evaluated by quantitation of vaginal Chlamydial load and by scoring the chronic pathological changes. The pre-challenge immuneresponse to the vaccine antigen will be accessed by quantitation of INFg after restimulation of spleen cells. and by assessing the serum antibody reactivity against a *C. trachomatis* EB lysate and the ELISA-reactivity against the immunogen.

The antigens checked in this model are: Ct015, Ct025, Ct026, Ct030, Ct048, Ct063, Ct078, Ct080, Ct184, Ct521, Ct051, Ct089, Ct175, Ct443, Ct456, Ct511, Ct541, Ct583 &.Ct603

Materials and methods.**Animals**

Female C57BL/6J, mice, 8-12 weeks of age, were obtained from Harlan Laboratories. Animals were housed under standard environmental conditions and provided standard food and water ad libitum. The use of mice is guided by the regulations set forward by the Danish ministry of justice (Lov om dyreforsøg, jvf lovbekendelser nr. 726 af 9. September 1993), and Animal protection committees. A detailed description of the proposed experiments has been submitted to and approved by the regional ethical review board (2003/561-786) held by the applicant.

Chlamydia muridarum

C. muridarum was propagated in HeLa 229 cells (ATCC, Rockville, MD, USA). The HeLa cells were grown in complete media (RPMI-1640 (Gibco BRL); 5% heat inactivated Fetal Bovine Serum (Cambrex bioscience); 1% v/v Hepes, 1% v/v L-glutamine, 1% v/v pyrovate and 10 µg/ml gentamycine. Subconfluent monolayers of HeLa 229 cells plated in 175 cm² flasks were pre-treated for 15 minutes at RT with 45 µg/ml DEAE-dextran in Hanks buffered salt solution (HBSS) and infected at an 1 MOI (i.e. one inclusion forming unit (IFU) of *C. muridarum* per HeLa cell) in 3 ml HBSS. After 2h of incubation at 37°C, 50 ml complete media supplemented with 5% glucose and 1 µg/ml cycloheximid were

added and the infected cells were further incubated for 42-44 hours in a humidified incubator containing 5% CO₂. After microscopically confirming the presence of inclusions within a proper amount of target cells the monolayer were dislodged from the flasks with a cell scraper and centrifuged 30min at 35.000g and 4°C. The pellets were resuspended in 5 ml HBSS per flask, sonicated on ice at 2 x 1000 joule and centrifuged at 500g for 15min 4°C. The supernatants were collected and stored on ice. The pellets were resuspended in 5 ml HBSS and sonicated and centrifuged as in the last step. The supernatants were pooled and centrifuged for 30min at 30.000g, 4°C and the pellets resuspended SPG buffer (250mM Sucrose; 10mM Na₂HPO₄; 5mM L-Glutamic acid). After a brief sonication the suspension was gently layered over a 30% Diatrizoate solution and centrifuged at 40,000g for 30min. After centrifugation the pellet were resuspended in SPG buffer and stored at -70°C.

Infectivity of the *C. muridarum* preparation was quantitated by titration on McCoy cells followed by enumeration of inclusions in immunofluorescence assay. Briefly, 90-95% subconfluent HeLa 229 monolayers were centrifuged for 1 hour at 750g at RT with titrated inoculum followed by incubation at for 2h at 35°C . The inoculum was replaced by complete medium supplemented with 5% glucose and 1 µg/ml cycloheximide and further incubated for 42-44h at 37°C. For staining the cells were fixed in 99% icecold ethanol for 15min. The fixed cells were incubated with a rabbit polyclonal anti-Chlamydia MOMP antibody for 1h followed by secondary staining with a FITC labelled swine-anti rabbit Ig antibody. The cells were counterstained with Propidium iodine. The inclusion positive cells in 20 high-power (40x) fields were enumerated with a fluorescence microscope to quantitate the infectivity of the *C. muridarum* stock (expressed in IFU/ul).

25

Infection of mice

Mice were infected by the intra vaginal route by 10⁵ to 10⁷ IFU's (100 – 10.000 ID₅₀). The infection was monitored at day 7 and day 14 after inoculation by obtaining cervicovaginal swabs followed by fluorescent staining and enumeration of infectious units in the specimen.

30

Immunization

Mice were immunized subcutaneously (sc) three times with 2 weeks interval at the base of the tail. The vaccines consisted of 1-5ug of peptide (see above) emulsified in 250ug DDA and 100ug TDB. As a negative control, DDA/TDB alone, without peptide were injected.

- 5 As a positive control, mice were infected intra nasally for 55-75 days with 10^5 IFU *C. muridarum*. The nasal infection leaves the animals almost completely protected, comparable to the protection induced by the vaginal infection.

10

Lymphocyte cultures, serum antibodies and evaluation of immuneinducing potential

- For evaluation of ability to induce a strong immuneresponse, spleens were taken at 21 days after last immunization and spleen lymphocytes were obtained by rubbing the tissue through a metal mesh to a single cell suspension, washed once in RPMI-1640 at 800g at
 15 RT and resuspended in re-stimulation media. (RPMI-1640, Gibo, 10% heat-inactivated Fetal Bovine Serum, Biochrom AG, Berlin, Penicillin G 100 U/ml, streptomycin 100ug/ml, 10 mM Hepes, 2 mM L-glutamine, 1 mM pyrovate).

- The isolated cells were cultured in triplicates in round-bottom 96-well plates at 2×10^5 cells
 20 per well in 200ul re-stimulation media. Peptides were added in concentrations ranging from 0.08 to 5ug /ml. and incubated for 72h. Negative and positive controls (either media or 5ug/ml ConA) were included in all experiments as necessary. After restimulation the supernatants were harvested and IFN- γ quantitated by enzyme-linked immunosorbent assay(Brandt, Elhay et al. 2000). Vaccine candidates giving high levels of critical IFNg
 25 above 2000 pg/ul was: Ct015, Ct025, Ct026, Ct030, Ct048, Ct063, Ct078, Ct080, Ct184, Ct521, native *C. Muridarum* MOMP, Ct051, Ct175, Cy443, Ct456 & Ct603, (Fig 16).

- At the same timepoint, blood samples were drawn from the eye sinus and serum prepared. Serum was tested for reactivity against *Chlamydia trachomatis* SvD and *Chlamydia muridarum* elementary bodies by western blot analysis (Theisen, Soe et al. 2004). Briefly,
 30 density gradient purified elementarybodies were electrophorezed on a 4-12% polyacrylamid gel, electro blotted onto nitrocellulose and blocked in skimmed milk.in a Mesh buffer. Pools of sera (4 animals from each vaccine group) were diluted 1:100 and

incubated with the blot for 1 hr, washed and further incubated with a secondary alkaline phosphatase coupled antibody for 1 hr. Reactions were visualized by incubation with BCIP/NBT (Sigma) substrate. Bands were evaluated as positives when observed size were in agreement with theoretical size. Positives were: Ct015, Ct030, Ct048, Ct078,
 5 Ct184 & Ct521 (Fig 15)

Serum was tested by ELISA (Rosenkrands, Agger et al. 2005) for reactivity against the recombinant protein used for immunization and against heat-inactivated *Chlamydia muridarum* elementary bodies. Briefly, plates were coated with antigen (0.5ug/ml) in
 10 carbonate buffer o/n, blocked with BSA and washed. The plates were incubated with pre-diluted samples for 2 hrs at room temperature, washed and incubated with a peroxidase conjugated secondary antibody for 1 hr. Reactions were visualized by incubation with TMB substrate and the reaction stopped with sulphuric acid and read at 450 nm. Titers at OD=1.0 were calculated after applying four-parameter fit on the data (Fig 14). Antigen
 15 high in IgG1 were: Ct015 & Ct030. Antigens high in IgG2b were: Ct063, Ct521High.

Evaluation of the protective efficacy

For evaluation of vaccine efficacy, mice were challenged 8-12 weeks after the first
 20 immunization by intra vaginal infection by 10^5 to 10^7 IFU's (100 – 10.000 ID₅₀). The protective efficacy of the vaccine candidates was monitored by pathological evaluation and by enumeration of infectious units obtained by cervicovaginal swabs.

25 The bacterial load was determined by cervicovaginal swabs obtained at 7, 14 and/or 21 days after challenge. The swabs were submerged in 1ml SPG buffer at 4°C until prepared. At the same day, *C. muridarum* EB's were mechanically shaken off of the swab by vortexing the specimen for 30s at full speed in the presence of mm glass beads. The buffer was transferred to eppendorf tubes and stored at -80°C until analyzed. Infectious EB's
 30 were quantitated by enumeration of inclusions in subconfluent McCoy cells in immunofluorescence assay as described above. (Figure 8) Antigens inducing protection after enumeration of swap-IFU's at PID7 are: Ct015, Ct025, Ct048, Ct184, Ct521, Ct443,

Ct603 and native *C. muridarum* MOMP.

For pathology whole genital tracts were evaluated macroscopically for signs of acute and chronic pathology at PID49.. From the gross pathological evaluation a hydrosalpinx-score
5 were calculated. The score is calculated as the ratio of hydrosalpinges over total number of fallopian tubes in the individual vaccine group (Figure 9). Antigens inducing a fair protection at PID42 are Ct025, Ct063, Ct184, Ct521.

Based on the available material, Ct184 and Ct521 are the antigens performing best in the
10 challenge model. Formulated in Lipovacc, they are inducing the least pathology and the best protection against vaginal Chlamydia.

15

Example 7: Screening for *C. trachomatis* specific T-cell epitope targets using the whole-genome random expression library

Introduction

The whole-genome random expression library was used for directly screening for potential
20 *C. trachomatis* specific antigen targets that stimulate T-cell proliferation in patient PBMC's. Pools of bacteria expressing random selected λ gt11 phages expressing recombinant polypeptides in fusion with β -galactosidase in the lysogen host bacteria Y1089r- (facilitating lysogen phage growth) are directly administered to patient PBMC cells. Following incubation, where the patient PBMC's are activated, possibly through
25 specific effector T-cells due to the bacterial expressed *C. trachomatis* antigen exposure, the mixture is cleared for further bacterial growth by adding antibiotics, and further incubated 2 to 4 days essentially as described in Example 1. The read out may be INF γ and/or specific T-cell proliferation.

30 In theory, a whole-genome random expression library containing individual random expressed *C. trachomatis* gene sequences of 0.4-0.8kb in size covers any gene sequence (in correct orientation and in reading frame with the fusion partner, β -galactosidase), in

about 1:10.000 individual lambda clones. Thus, screening of 10 to 20 pools each containing 500 to 1000 of randomly selected bacterial clones covers the whole *C. trachomatis* genome. Data by Alderson et al (2000) have shown that as much as adding 10^6 control bacteria/well containing as little as 10^4 T cells result in low level of unspecific INF γ and proliferation. Significant and specific INF γ release as well as specific T-cell proliferation was found by adding as little as 10^3 antigen specific bacteria/well containing as little as 10^4 T-cells. Thus, a pool with 10^6 bacteria containing 500 different individual clones added to 10^5 PBMC cells/well may expose the T-cell population in each well with 2000 bacteria specifically expressing a particular recombinant fusion.

Materials and methods

Construction of the λ gt11 phage clone expressing β -galactosidase/CT521 fusion.

A λ gt11- β gal/CT521 was constructed for use as positive T-cell epitope target control. The full length sequence encoding the CT521 was amplified by PCR using *C. trachomatis* serovar D genomic DNA as template and the specific forward primer, 5'-TATAGAATTCATGTTAATGCCTAAACGAACAAAA- 3', and reverse primer, 5'-TATAGAATTCTTATACCCTTTCCACACGCTTAACAAATCG -3', containing EcoRI sites for cloning into the EcoRI cloning site of the λ gt11 expression vector in frame with β -galactosidase open reading frame. The cloned recombinant phage construct was verified for correct orientation and sequence by direct sequencing individual phage plaques (see example 3).

Preparation of whole-*Chlamydia trachomatis*-genome random expression library as λ gt11 lysogen library.

The lysogen bacterial stock of the whole- *C. trachomatis* -genome random expression library in Y1089r- is essentially constructed by the method described by Singh et al (1989).

Example 8

PBMC from 10 Chlamydia patients and 5 controls were isolated and cultivated as described previously (Example 1). Cell cultures were established in triplicate cultures of 1.25×10^5 PBMCs and stimulated with 5 μ g of protein. Cell cultures without antigen were included as negative controls (C), and PHA (2 μ g/ml) was used as a mitogenic positive control (result not shown). The following antigens were tested : CT043, CT008 CT016 CT025 CT026, CT048, CT098, CT110, CT125, CT155, CT003, CT005, CT023, CT027, CT028, CT032, CT035, CT078, CT082, CT093, CT111, CT123, CT126, CT133, CT175, CT184, CT002, CT009, CT015, CT061, CT063, CT068, CT071, CT080; CT089, CT141, CT509, CT803, CT004, CT030, CT038, CT040, CT052, CT053, CT067, CT511, CT583, CT603, CT681, CT265, CT323, CT322, CT342, CT357r, CT375, CT376, CT456, CT213, CT168, CT396, CT443, CT587, CT610, CT679, CT842, CT875, CT561, CT659, CT112, CT124, CT150, CT201, CT245, CT246, CT405, CT420, CT426, CT507, CT512, CT513, CT514, CT516, CT316, CT439, CT492, CT520, CT523, CT526, CT611, CT613, CT626, CT630, CT647, CT649, CT725, CT734, CT779, CT 801, CT833, CT835, CT836, CT845 and CT541 (Fig. 10).

As seen in figure 10 the degree of human recognition varies. Some are strongly and frequently recognized - more than 5 patients responding with a level of IFN- γ above all controls. These includes CT375, CT376, CT004, CT048, CT078, CT110, CT583, CT603, CT681, CT184, CT175, CT025, CT002, CT015, CT063, CT456, CT168, CT396, CT443, CT124, CT028, CT030, CT43, CT048, CT080, CT111, CT316, CT322, CT342, CT375, CT492, CT512, CT520, CT521, CT523, CT541, CT611, CT613, CT630, CT649, CT734, CT801, CT803 (Figure 11) whereas others are not recognized at all (ex. CT071, CT133, CT005).

Example 9

Mapia testing of antibody targets - essentially as described in Lyashchenko et. al. (2000).
Briefly, antigens from example 2 and 3 were purified as described in example 1. Antigens
were printed on nitrocellulose membrane and tested for reaction against a panel of patient
sera and control sera (20 of each). Controls were used for defining the visual cut-off.
Patient sera with a clear reaction over the visual cut-off are regarded as positive and are

ranked from 1 to 20 positives.

Antigen	Number of Positive
Ct051	4
Ct080	0
Ct089	10
Ct110	18
Ct115	1
Ct118	6
Ct119	9
Ct125	8
Ct141	0
Ct155	0
Ct168	7
Ct174	0
Ct184	1
Ct283	1
Ct396	5
Ct443	19
Ct456	8
Ct541	9
Ct643	0
Ct681	19
Ct842	2
Ct874	4

5

Example 10: Protection strategy in C3H/HeN mice

The antigens examined in this model are: CT521, TC0052 (muridarum major outer membrane protein) and the combination of the two proteins.

10

Materials and methods.

Animals

Female C3H/HeN mice, 8-12 weeks of age, were obtained from Harlan Laboratory. Animals were housed under standard environmental conditions and provided standard food and water ad libitum

5

Chlamydia muridarum

C. muridarum was propagated in HeLa 229 cells and harvested as described in Example 6.

Infection of mice

- 10 Mice were infected by the intravaginal route by 10^5 IFU's. The infection was monitored at day 7 day 14 and day 21 after inoculation by obtaining cervicovaginal swabs followed by fluorescent staining and enumeration of infectious units in the specimen as described in example 6.

Immunization

- 15 Mice were immunized subcutaneously (sc) three times with 2 weeks interval at the base of the tail. The vaccines consisted of either 5 μ g rCT521, 5 μ g rTC0052 or the combination (5 μ g rCT521 + 5 μ g rTC0052) emulsified in 250ug DDA and 100ug TDB. As a negative control, DDA/TDB alone, without protein was injected.

20 Lymphocyte cultures and evaluation of immuneinducing potential

- For evaluation of ability to induce a strong immune response , blood samples were drawn from the eye sinus 7 days after the last immunization, pooled in groups (10 mice) and the blood lymphocytes purified on density gradient and resuspended in re-stimulation media (RPMI-1640, Gibo, 10% heat-inactivated Fetal Bovine Serum, Biochrom AG, Berlin,
25 Penicillin G 100 U/ml, streptomycin 100ug/ml, 10 mM Hepes, 2 mM L-glutamine, 1 mM pyrovate).

- The isolated cells were cultured in triplicates in round-bottom 96-well plates at 2×10^5 cells per well in 200ul re-stimulation media. Proteins were added in concentrations ranging from 0.31ug/ml to 10 μ g/ml and incubated for 72h. Negative and positive controls (either
30 media or 5ug/ml ConA) were included. After restimulation the supernatants were harvested and IFN- γ quantitated by enzyme-linked immunosorbent assay (Brandt et al., 2000) (Figure 12). Immunization with rCT521 induced a strong IFN- γ release in response

to restimulation with rCT521 and an epitope mapping (peptides described in example 1) of CT521 revealed P4 (aa 40-62) as the dominant epitope (Figure 12a). Likewise immunization with rTC0052 also induced a strong release of IFN- γ in response to the homologous protein (Figure 12b). Interestingly the mixture of rCT521 and rTC0052 very
5 efficiently enhanced the response to rCT521 compared to immunization with rCT521 alone (Figure 12c).

Evaluation of the protective efficacy

For evaluation of vaccine efficacy, mice were challenged 10 weeks after the first immunization by intravaginal infection with 10^5 IFU's. The protective efficacy of the
10 vaccine candidates was monitored by enumeration of infectious units obtained by cervicovaginal swabs as described in example 6. Both rCT521 and rTC0052 induced high levels of protection and the combination of the two proteins had a positive additive effect on protection (Figure 13). Protection experiments with rCT521 have been repeated in the C3H/HeN mice with similar results and high levels of protection after immunisation with
15 rCT521 has also been found in BALB/c x C57BL/6j F1 mice (results not shown).

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Claims

1. Use of a substantially pure polypeptide, which comprises an amino acid sequence selected from:
 - (a) SEQ ID NO. 237;
 - (b) a fragment of the sequence in (a) comprising a B-cell or a T-cell epitope; and
 - (c) an amino acid sequence analogue having at least 90% sequence identity to any one of the sequences in (a) and (b) and at the same time comprising a B-cell or a T-cell epitope,or use of the nucleic acid encoding the polypeptide of (a), (b) or (c),
for preparing a pharmaceutical composition for preventing or treating infections caused by a bacteria from a Chlamydia species,
wherein the polypeptide is fused to a fusion partner.
2. The use according to claim 1, where the polypeptide is lipidated so as to allow a self-adjuvating effect of the polypeptide.
3. The use according to any one of claims 1-2, where the fusion partner comprises:
 - (a) a polypeptide from a Chlamydia species, or
 - (b) at least one fragment of any of the polypeptides in (a) comprising a B-cell or a T-cell epitope.
4. The use according to any one of claims 1-2, where the nucleic acid sequence encoding the polypeptide in (a) has the sequence set forth in SEQ ID NO. 238.
5. A pharmaceutical composition for preventing or treating infections caused

by a bacteria from a Chlamydia species, comprising a substantially pure polypeptide, which comprises an amino acid sequence selected from:

- (a) SEQ ID NO. 237;
- (b) a fragment of the sequence in (a) comprising a B-cell or a T-cell epitope; and
- (c) an amino acid sequence analogue having at least 90% sequence identity to any one of the sequences in (a) and (b) and at the same time comprising a B-cell or a T-cell epitope,

or the nucleic acid encoding the polypeptide of (a), (b) or (c),

wherein the polypeptide is fused to a fusion partner.

6. The pharmaceutical composition according to claim 5, where the polypeptide is lipidated so as to allow a self-adjuvating effect of the polypeptide.

7. The pharmaceutical composition according to claim 5, where the fusion partner comprises:

- (a) a polypeptide from a Chlamydia species, or
- (b) at least one fragment of any of the polypeptides in (a) comprising a B-cell or a T-cell epitope; or

the nucleic acid encoding the polypeptide of (a) or (b).

8. The pharmaceutical composition according to claim 5 where the nucleic acid sequence encoding the polypeptide in (a) has the sequence set forth in SEQ ID NO. 238.

9. The pharmaceutical composition according to any one of claims 5-8, which is in the form of a vaccine against Chlamydia infection.

10. The vaccine according to claim 9 for immunizing against or treating

infections caused by a bacteria from a Chlamydia species.

11. The vaccine according to claim 10, where the Chlamydia species is *C. trachomatis*.

12. A monoclonal antibody or a fragment thereof which specifically binds to a polypeptide comprising an amino acid sequence selected from:

- (a) SEQ ID NO. 237;
- (b) a fragment of the sequence in (a) comprising a B-cell or a T-cell epitope;
and
- (c) an amino acid sequence analogue having at least 90% sequence identity to any one of the sequences in (a) and (b) and at the same time comprising a B-cell or a T-cell epitope
in an immunoassay.

13. A method for diagnosing previous or ongoing infection by a bacterium from a Chlamydia species, said method comprising contacting a sample *in vitro* with a diagnostic reagent in order to detect a positive reaction, wherein said diagnostic reagent comprises a substantially pure polypeptide, which comprises an amino acid sequence selected from:

- (a) SEQ ID NO. 237;
- (b) a fragment of the sequence in (a) comprising a B-cell or a T-cell epitope;
and
- (c) an amino acid sequence analogue having at least 90% sequence identity to any one of the sequences in (a) and (b) and at the same time comprising a B-cell or a T-cell epitope;

or the nucleic acid encoding the polypeptide of (a), (b) or (c).

14. An *in vitro* method for diagnosing previous or ongoing infection by a

bacterium from a Chlamydia species, said method comprising:

contacting a sample with the antibody according to claim 12 and detecting the antibody, wherein a positive reaction indicates infection.

15. An *in vitro* method of diagnosing previous or ongoing infection with a bacterium from a Chlamydia species in a subject comprising:

(a) contacting a diagnostic reagent with a bodily fluid of the subject, wherein said diagnostic reagent comprises a substantially pure polypeptide, which comprises an amino acid sequence selected from:

(1) SEQ ID NO. 237;

(2) a fragment of the sequence in (1) comprising a B-cell or a T-cell epitope; and

(3) an amino acid sequence analogue having at least 90% sequence identity to any one of the sequences in (1) and (2) and at the same time comprising a B-cell or a T-cell epitope;

or the nucleic acid encoding for the polypeptide of (1), (2) or (3); and

(b) detecting binding of an antibody to said polypeptide, said binding being an indication that said subject is infected by the bacterium from the Chlamydia species.

Figure 1

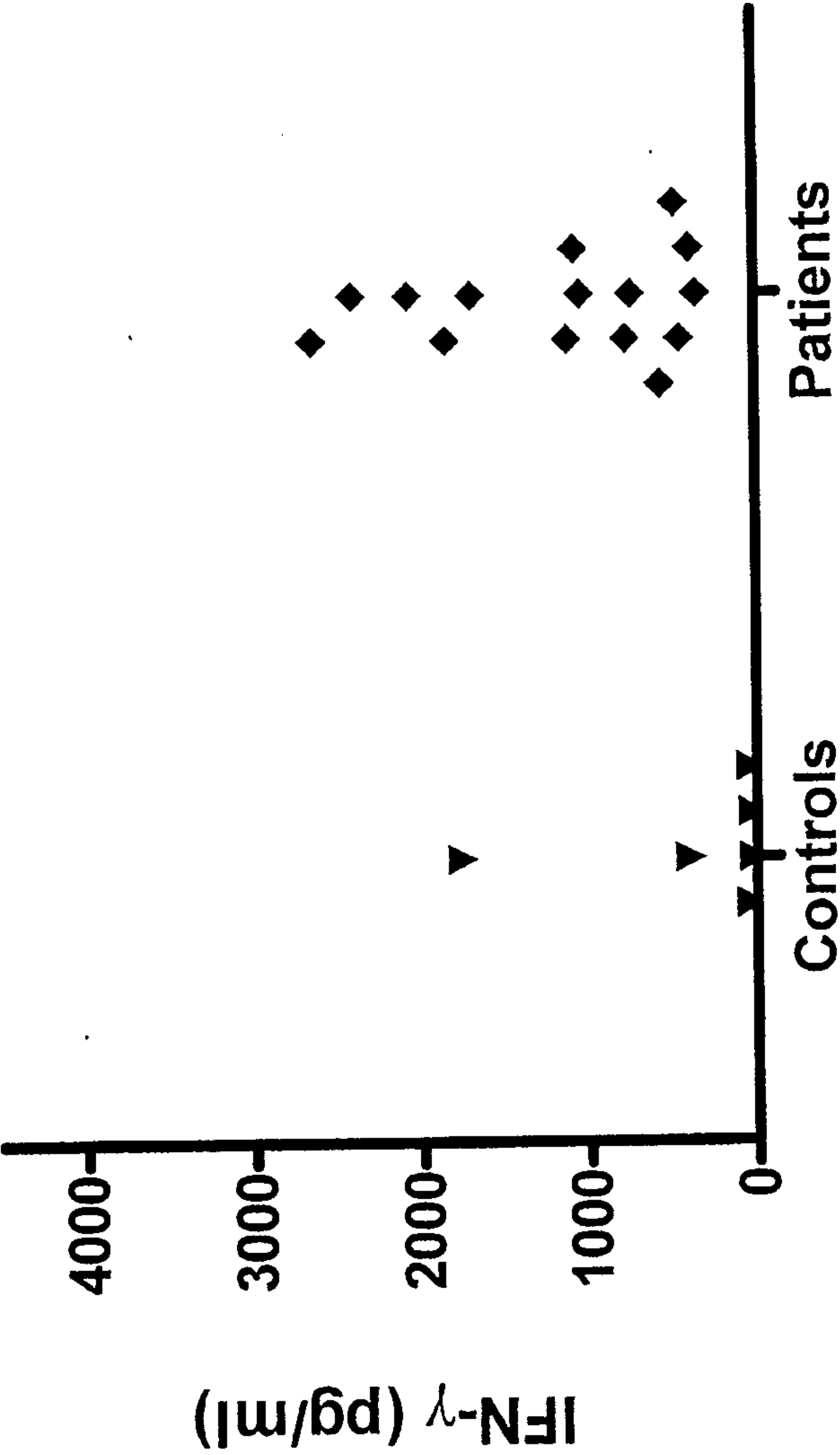


Figure 2



Figure 3



Figure 4

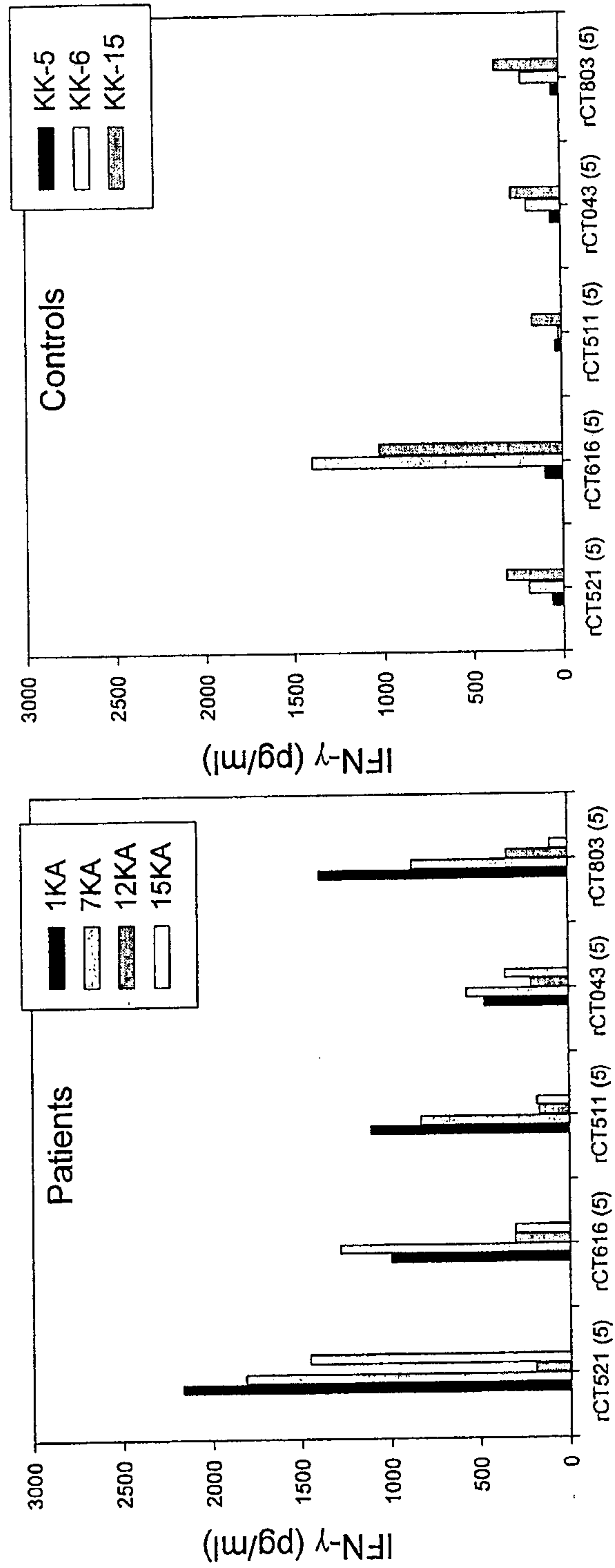


Figure 6

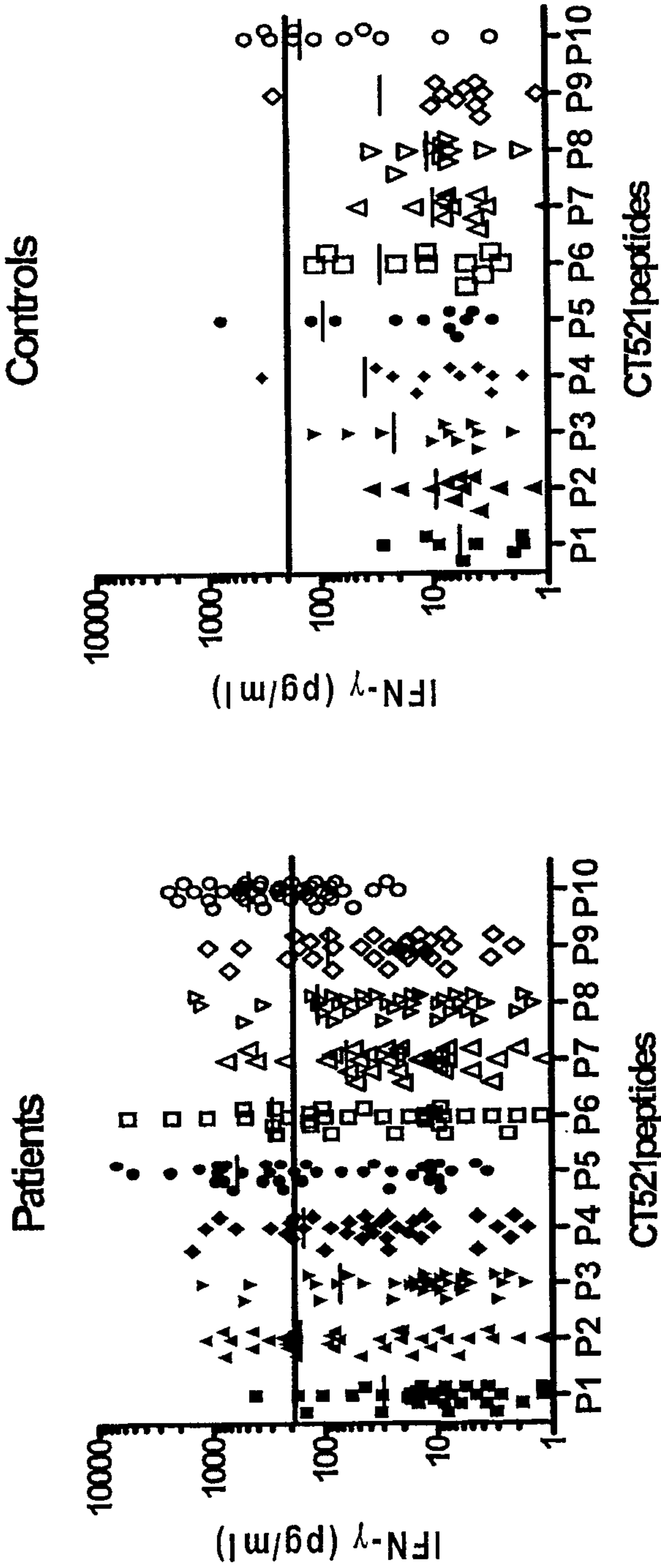


Fig 8a

Vaccination trial (7 days post-challenge)

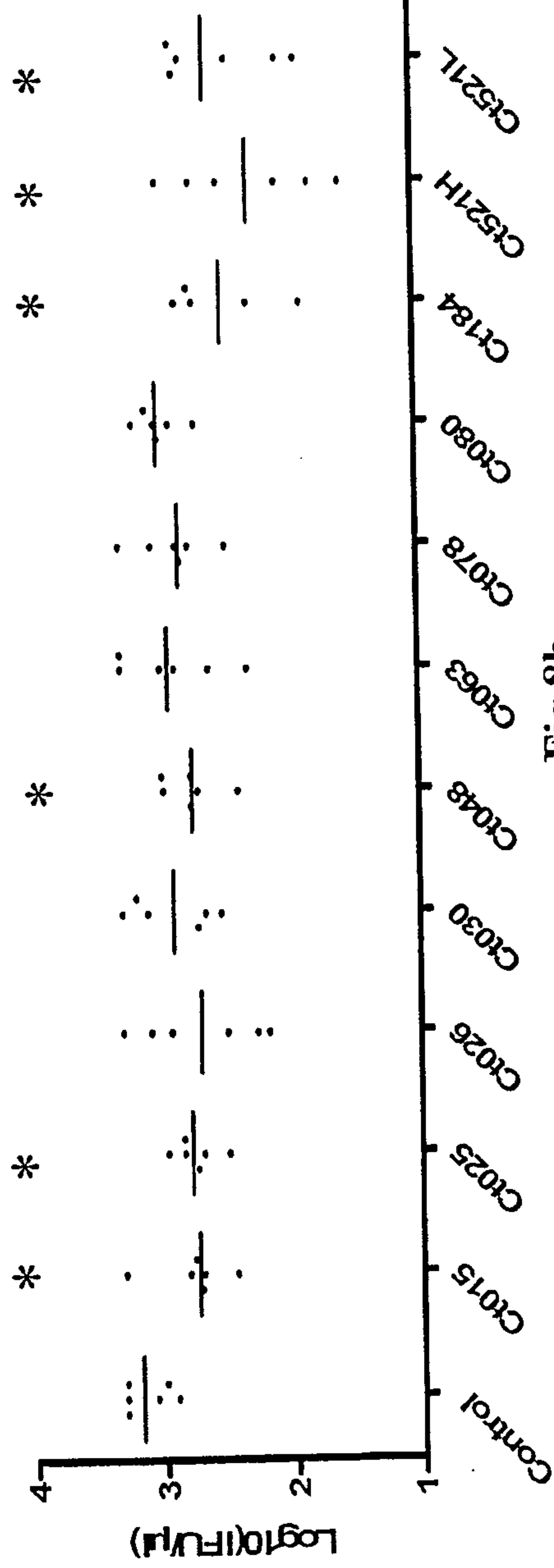
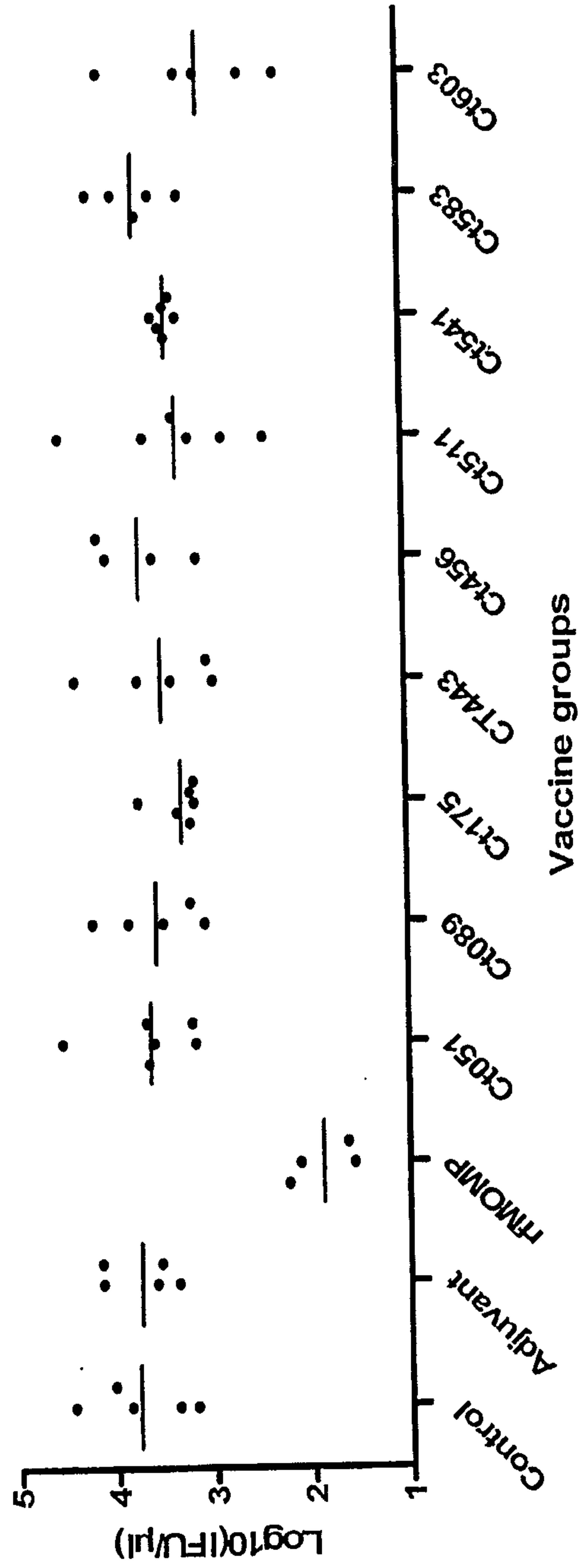


Fig 8b



* p<0.05

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Fig 8c

Vaccination trial (14 days post-challenge)

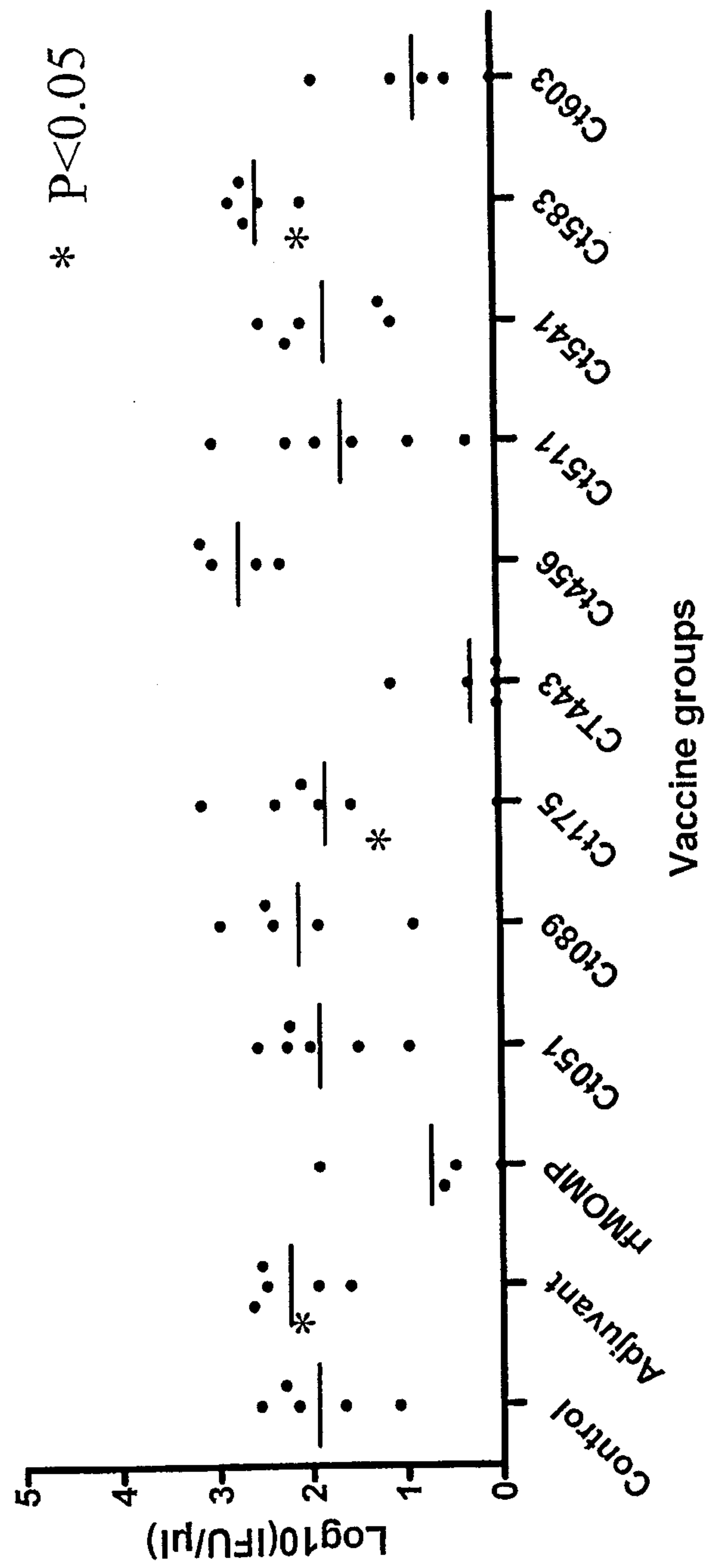
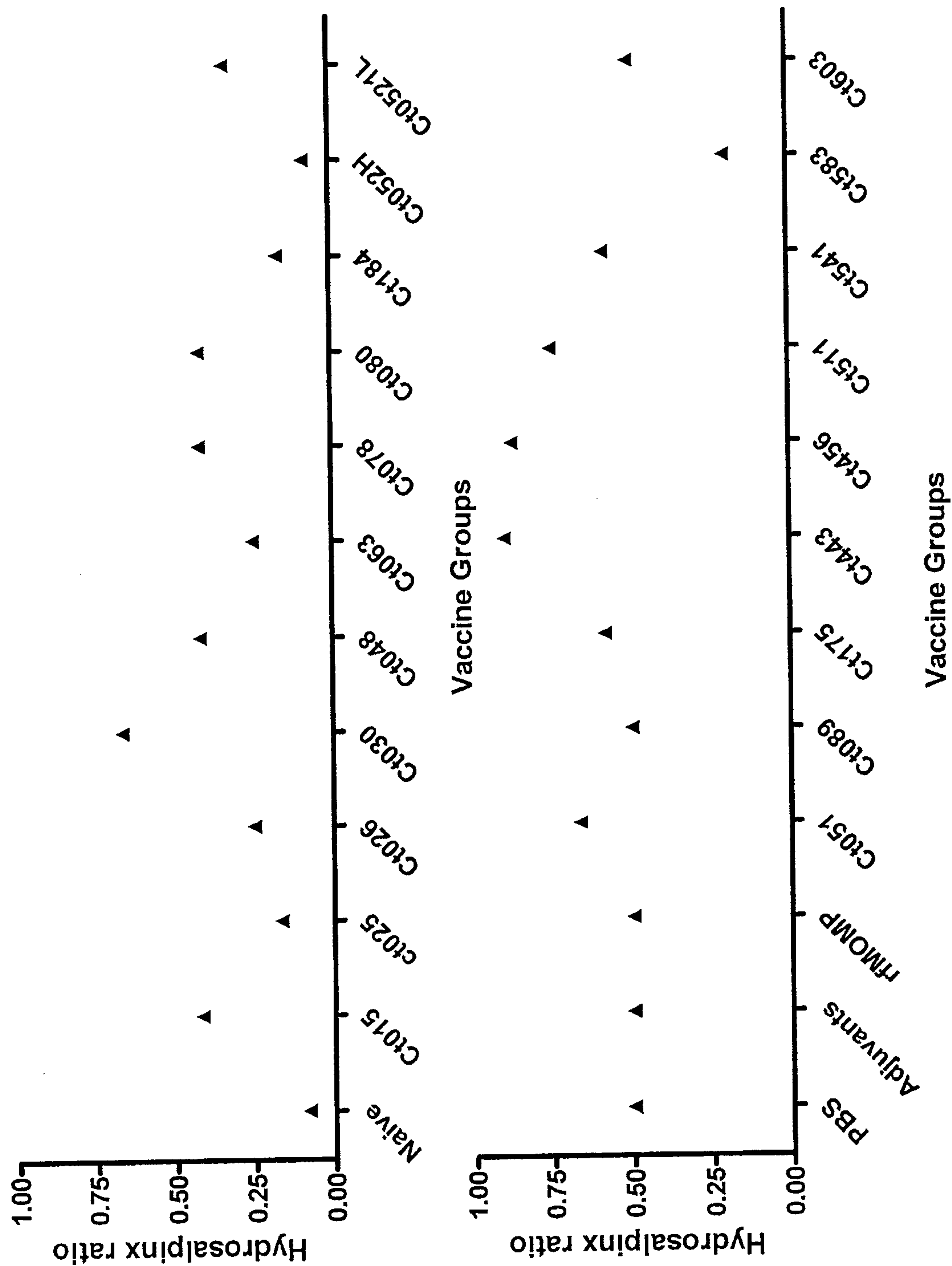
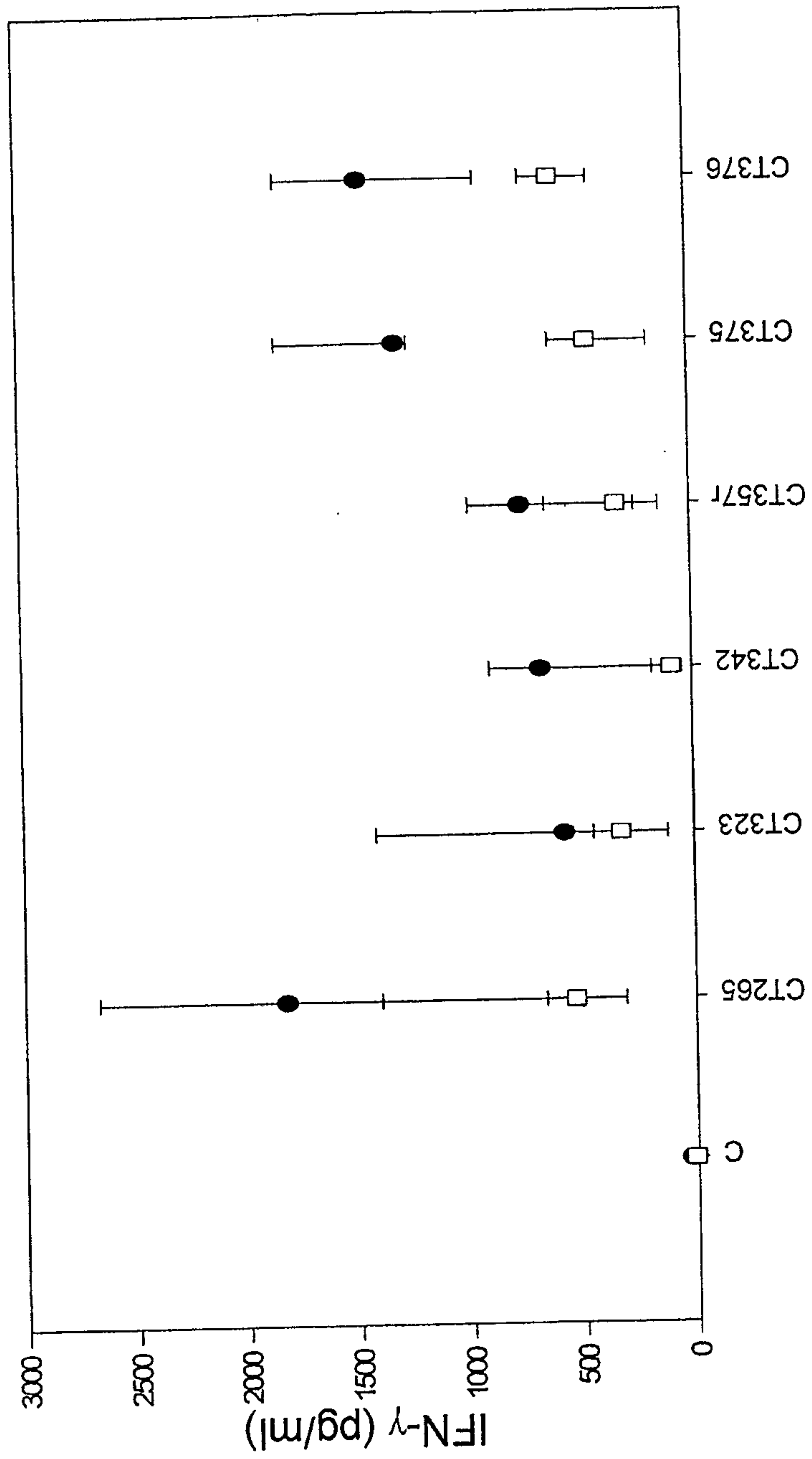


Fig 9 Hydrosalpinx ratio (49 days post challenge)



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Figure 10a



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Figure 10b

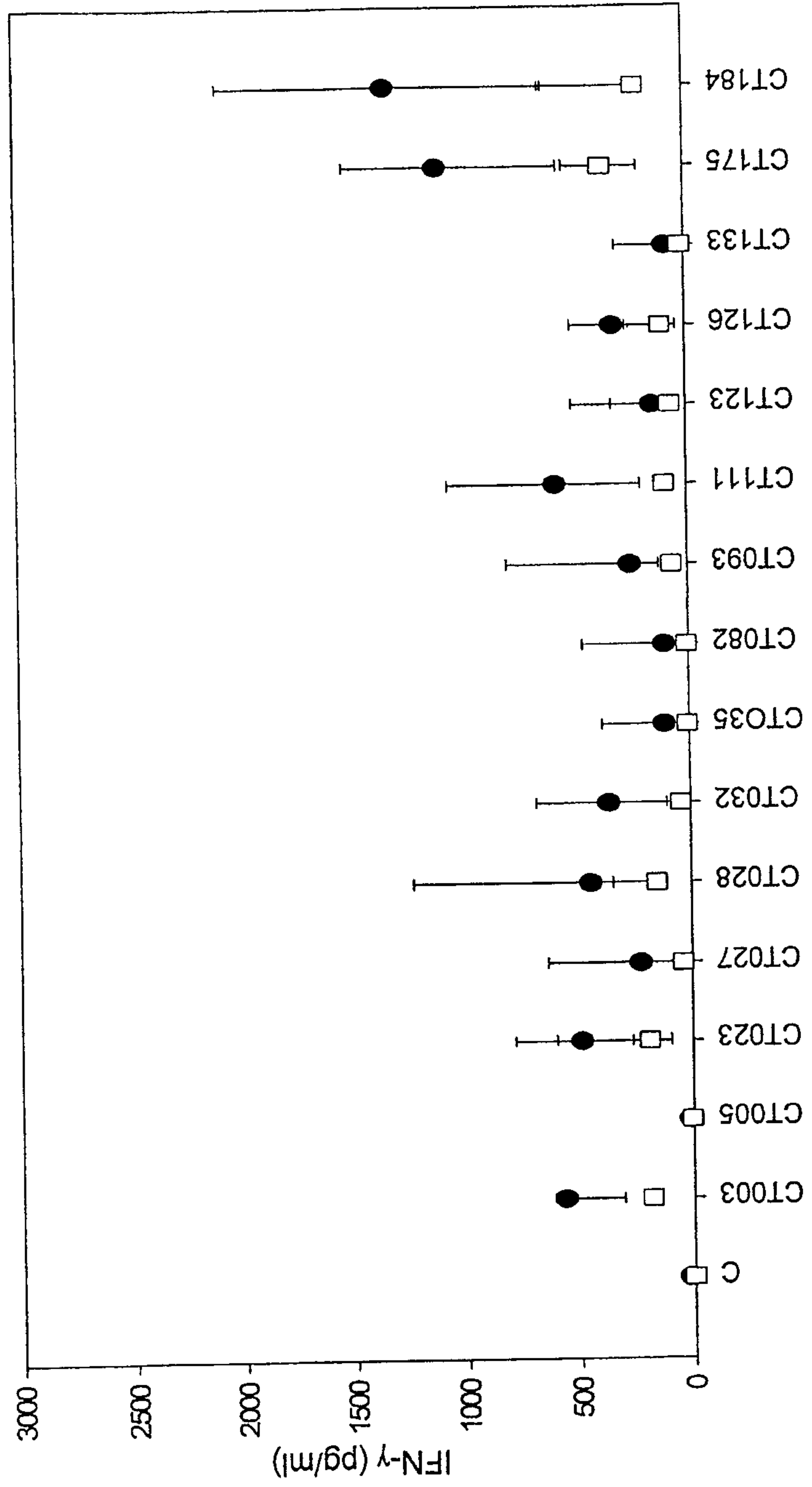


Figure 10c

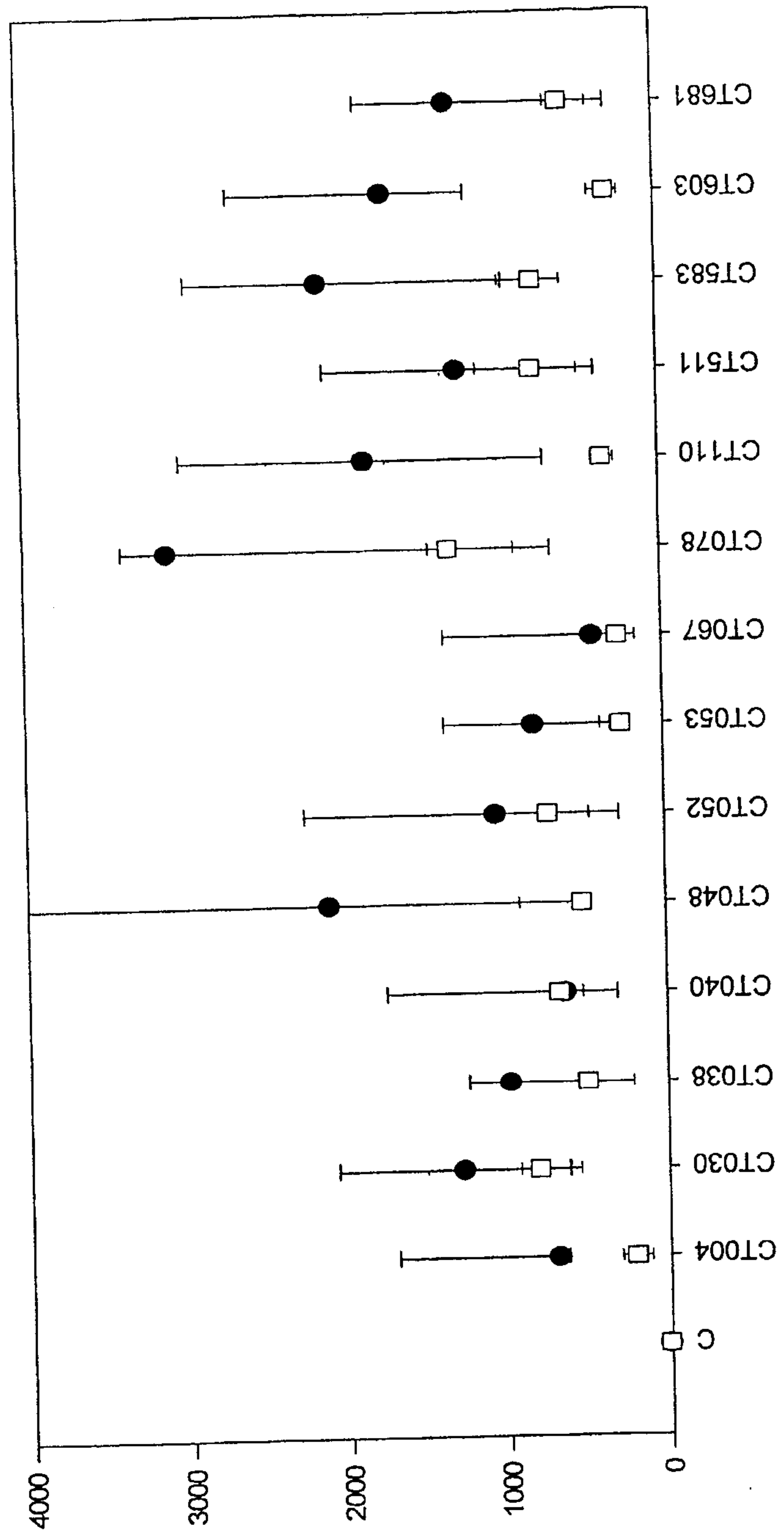


Figure 10d

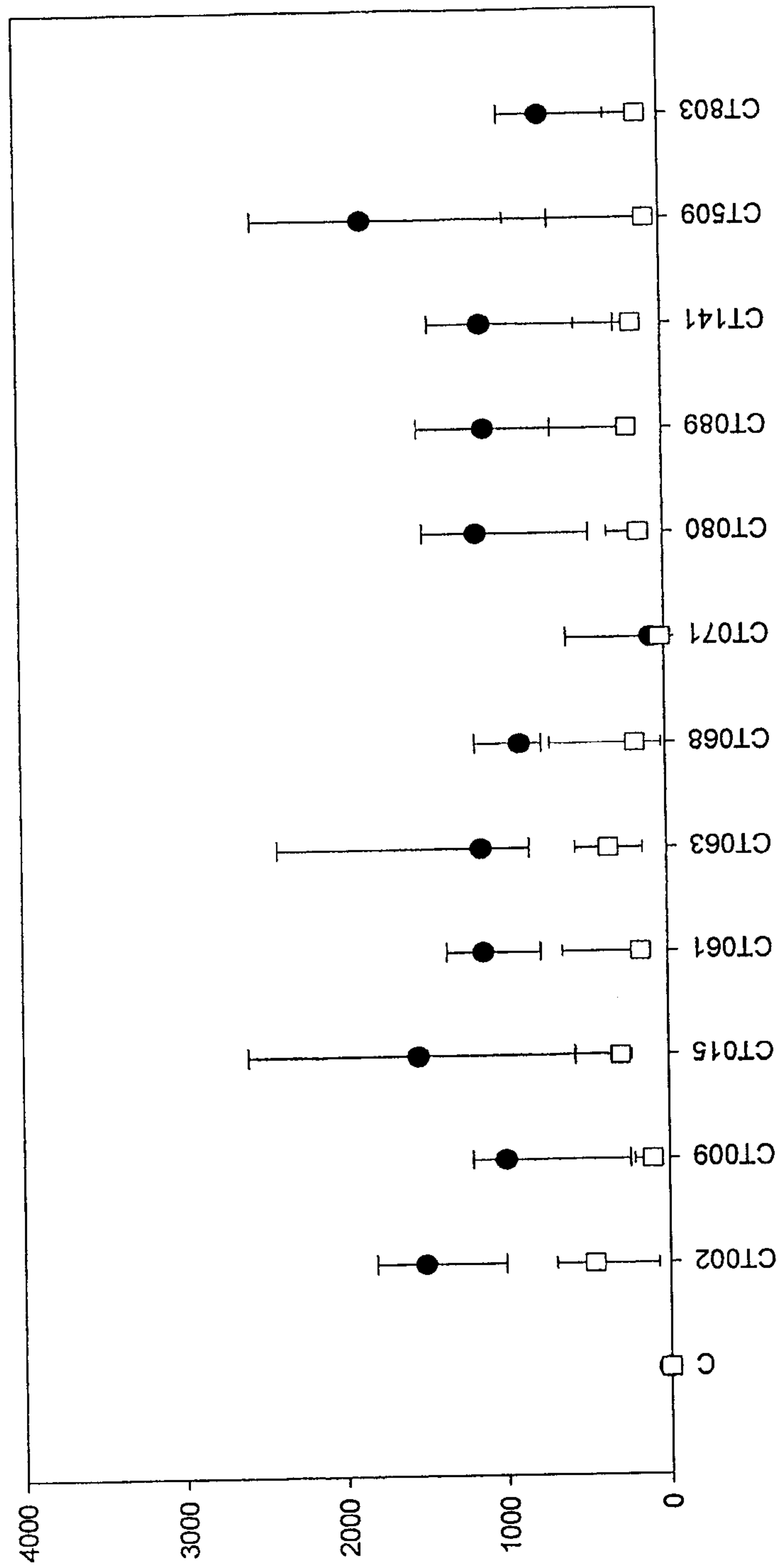


Figure 10e

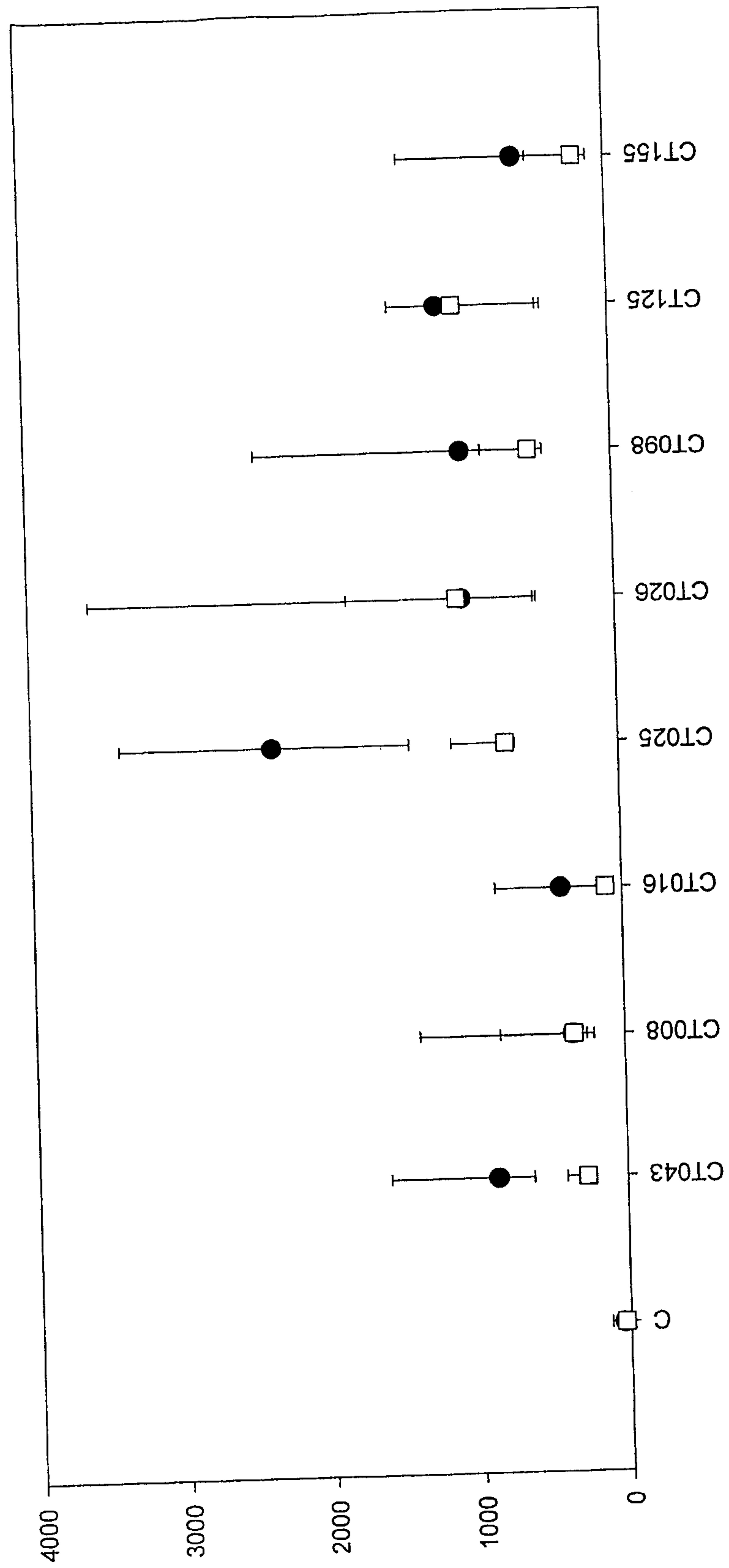


Figure 10f

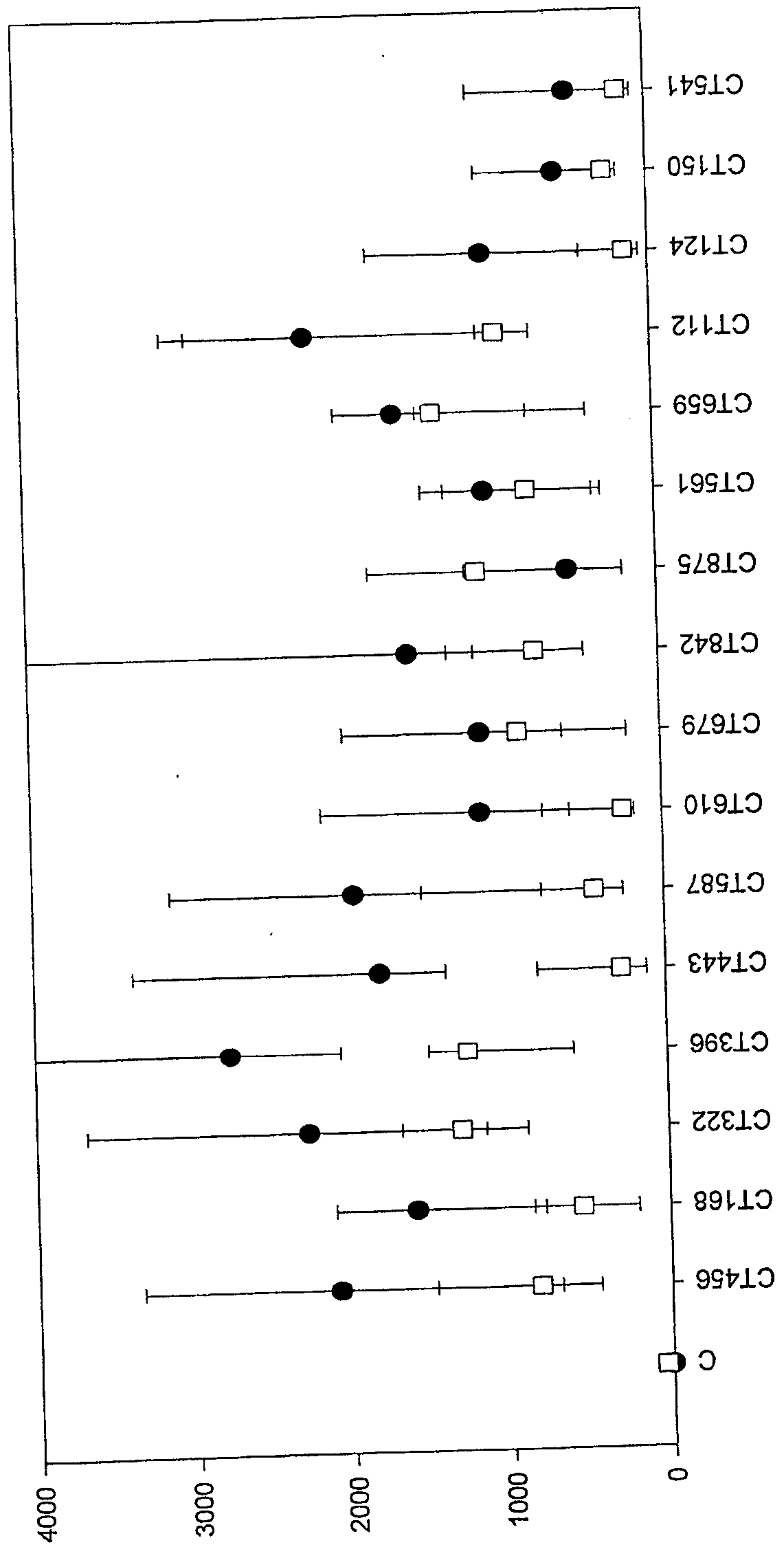


Figure 10g

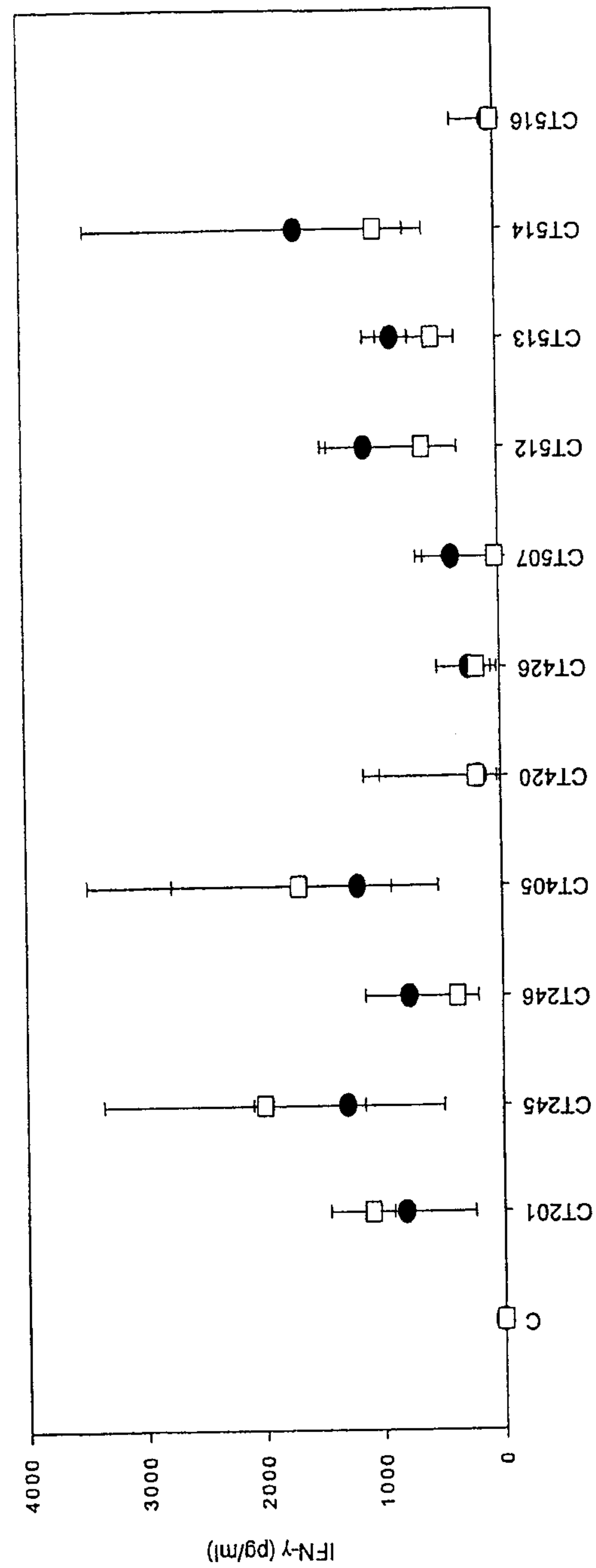


Figure 10h

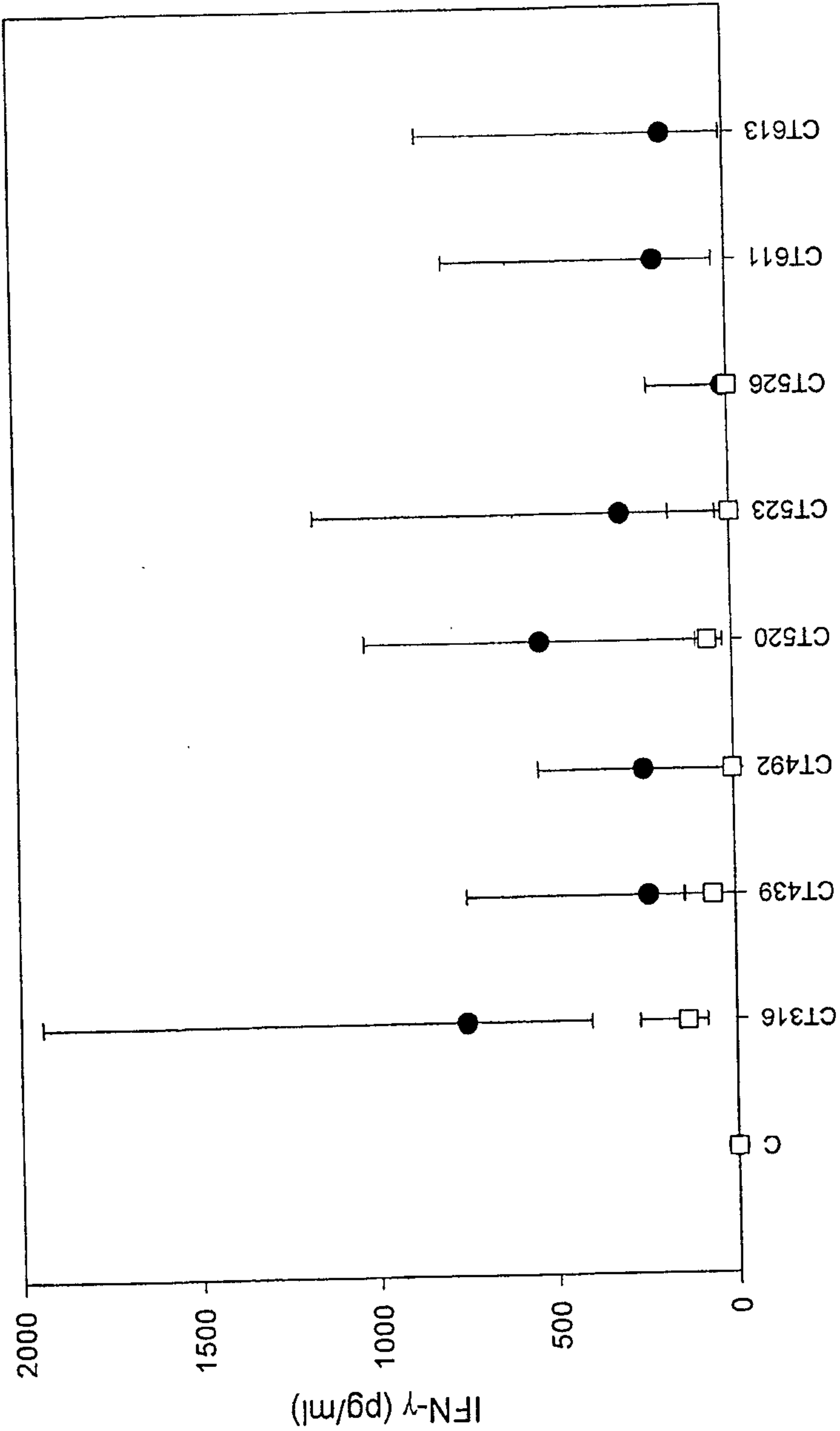


Figure 10i

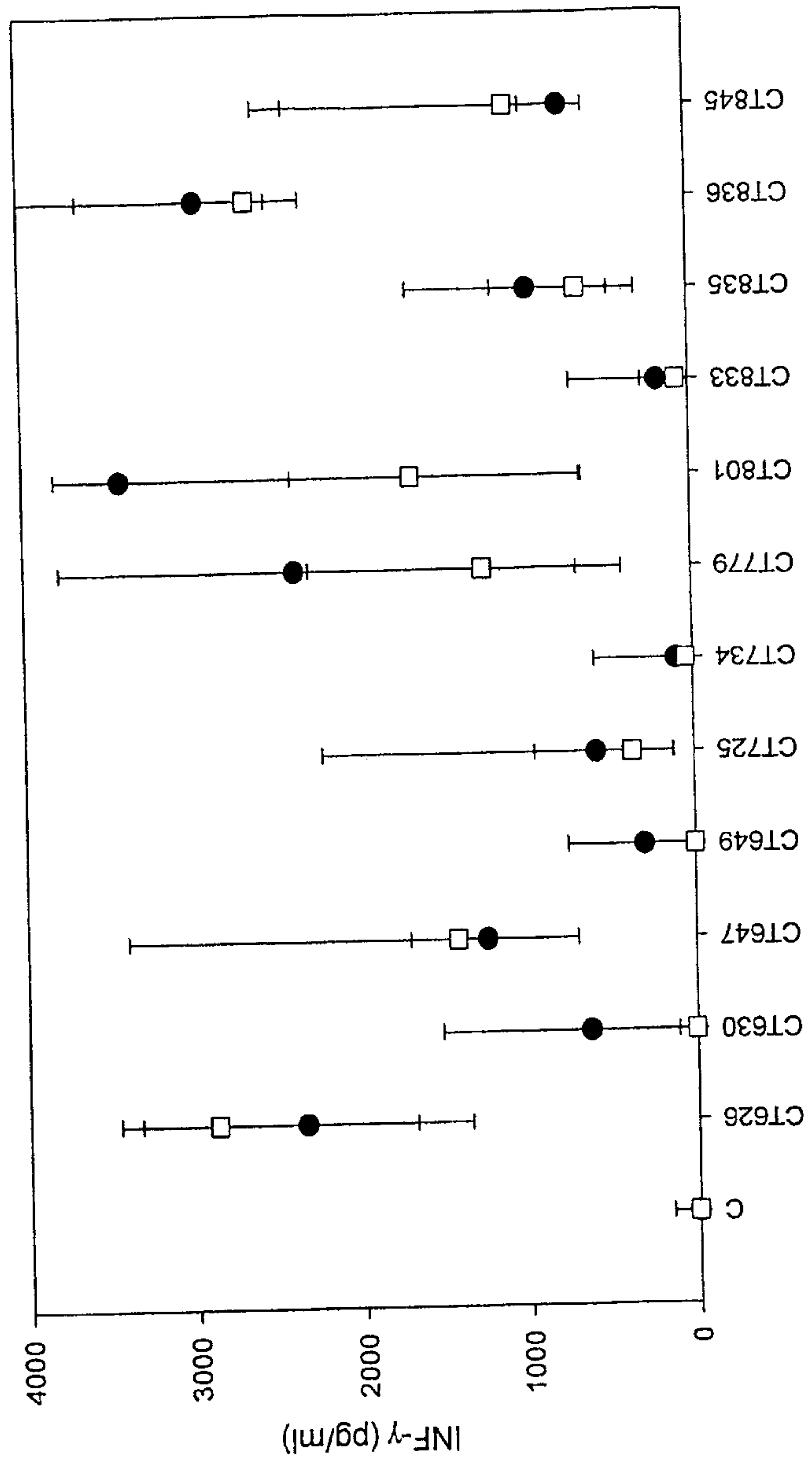


Figure 11

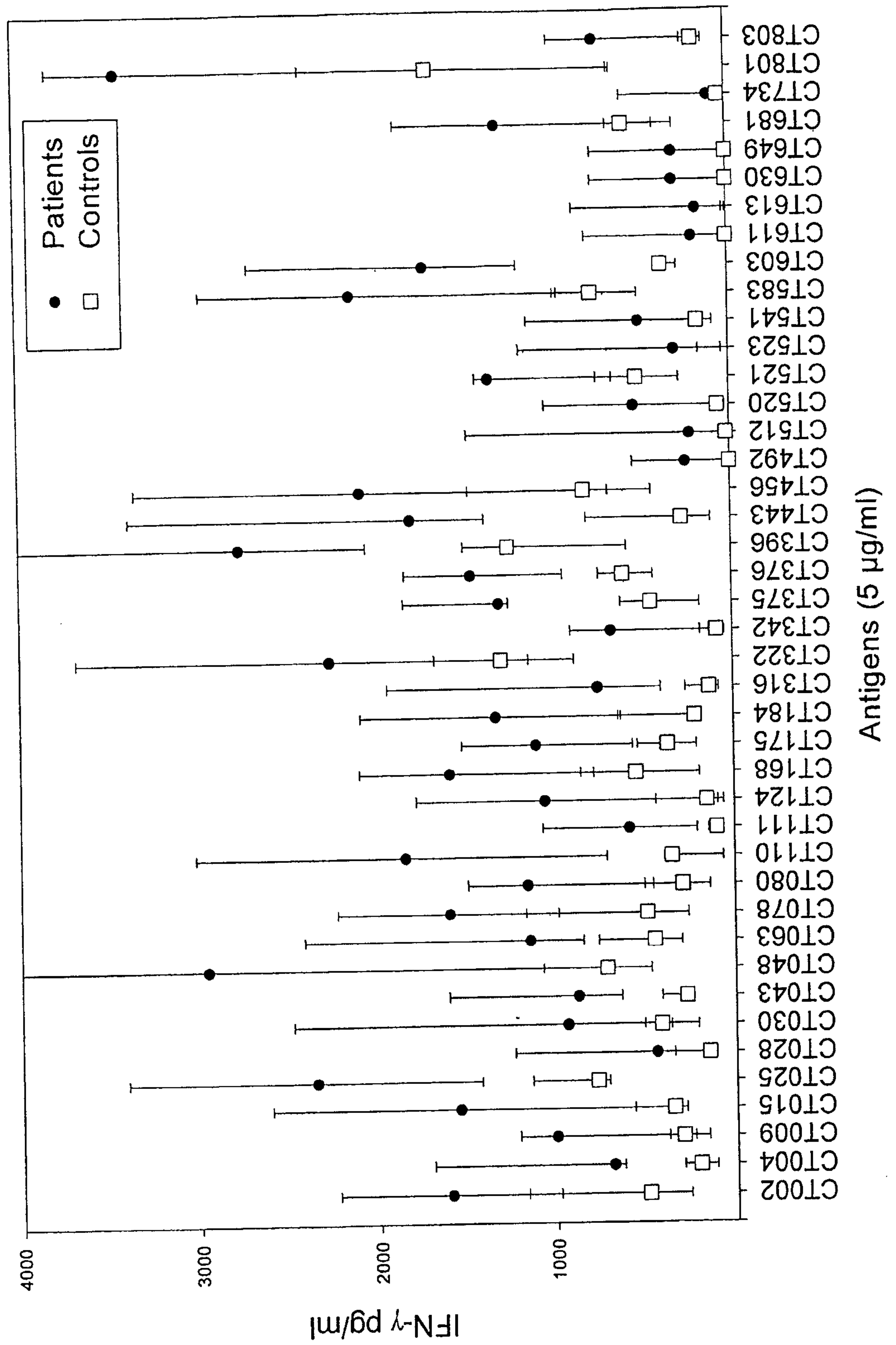
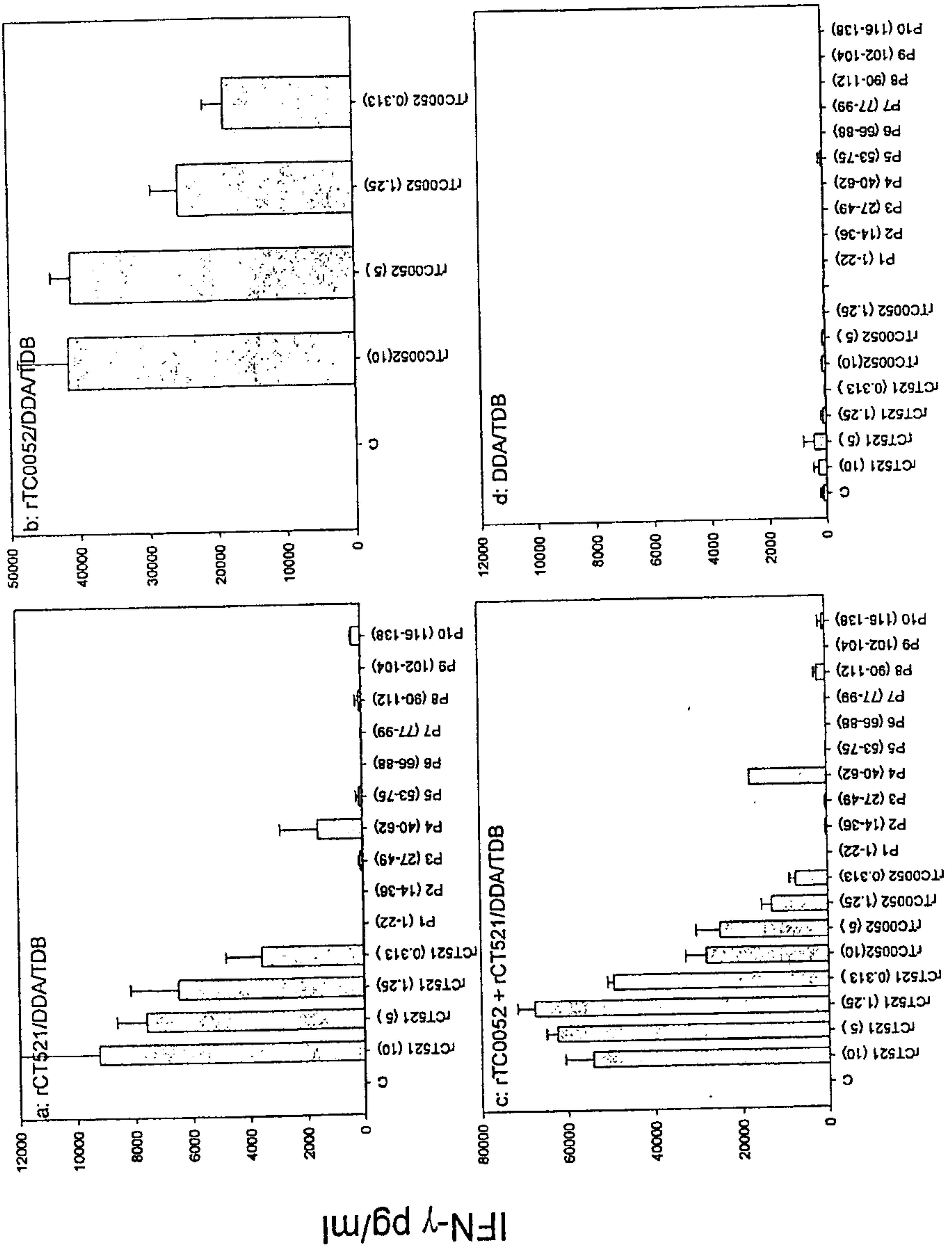


Figure 12



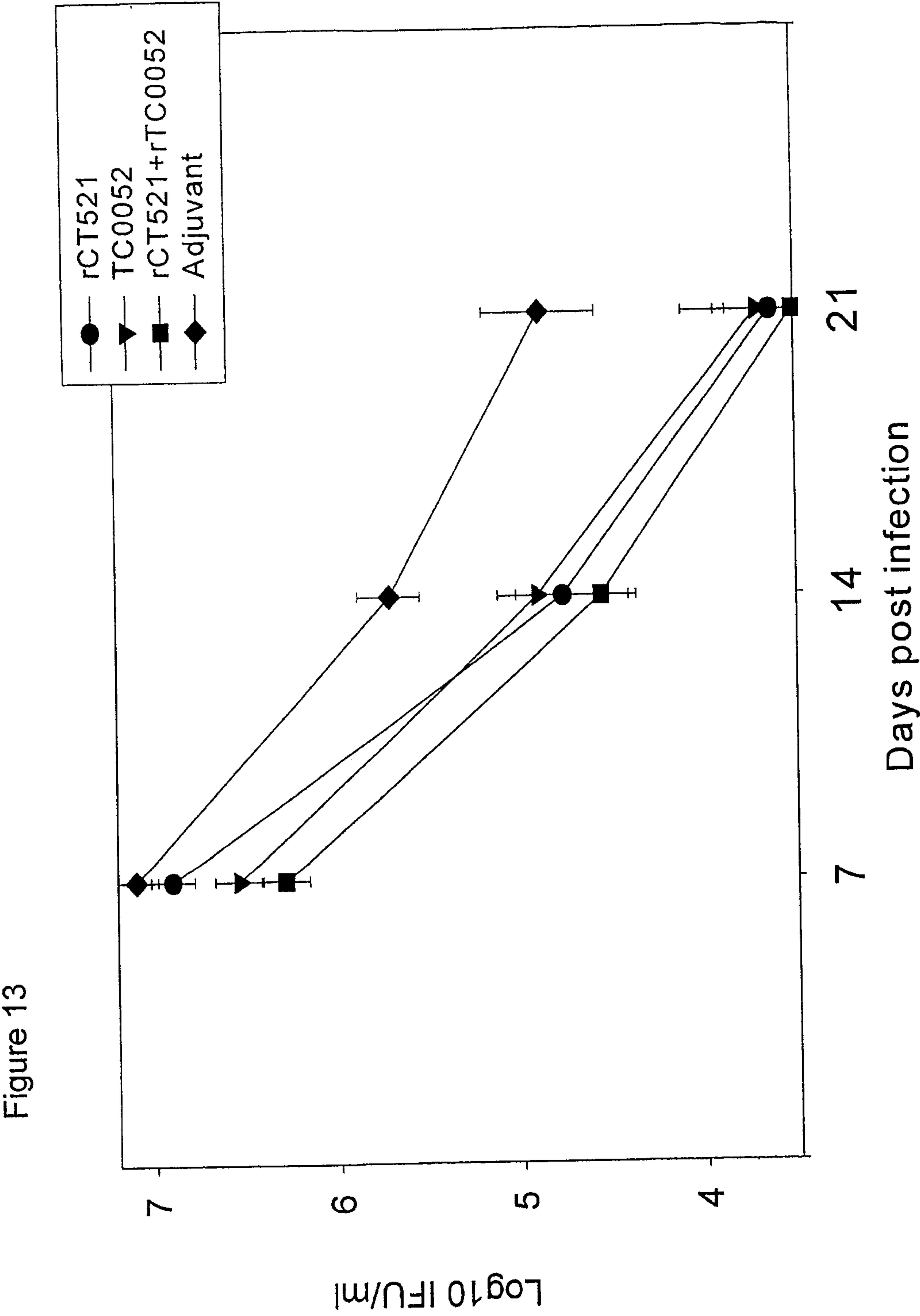
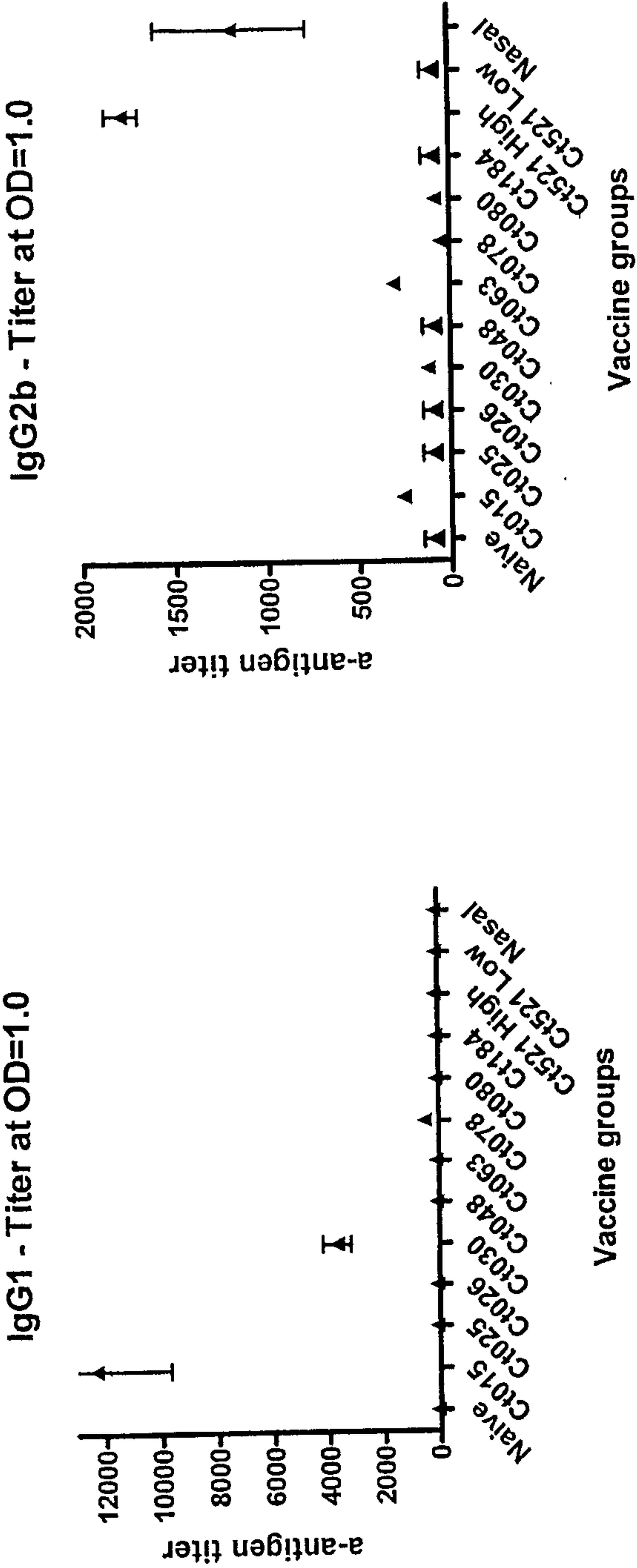
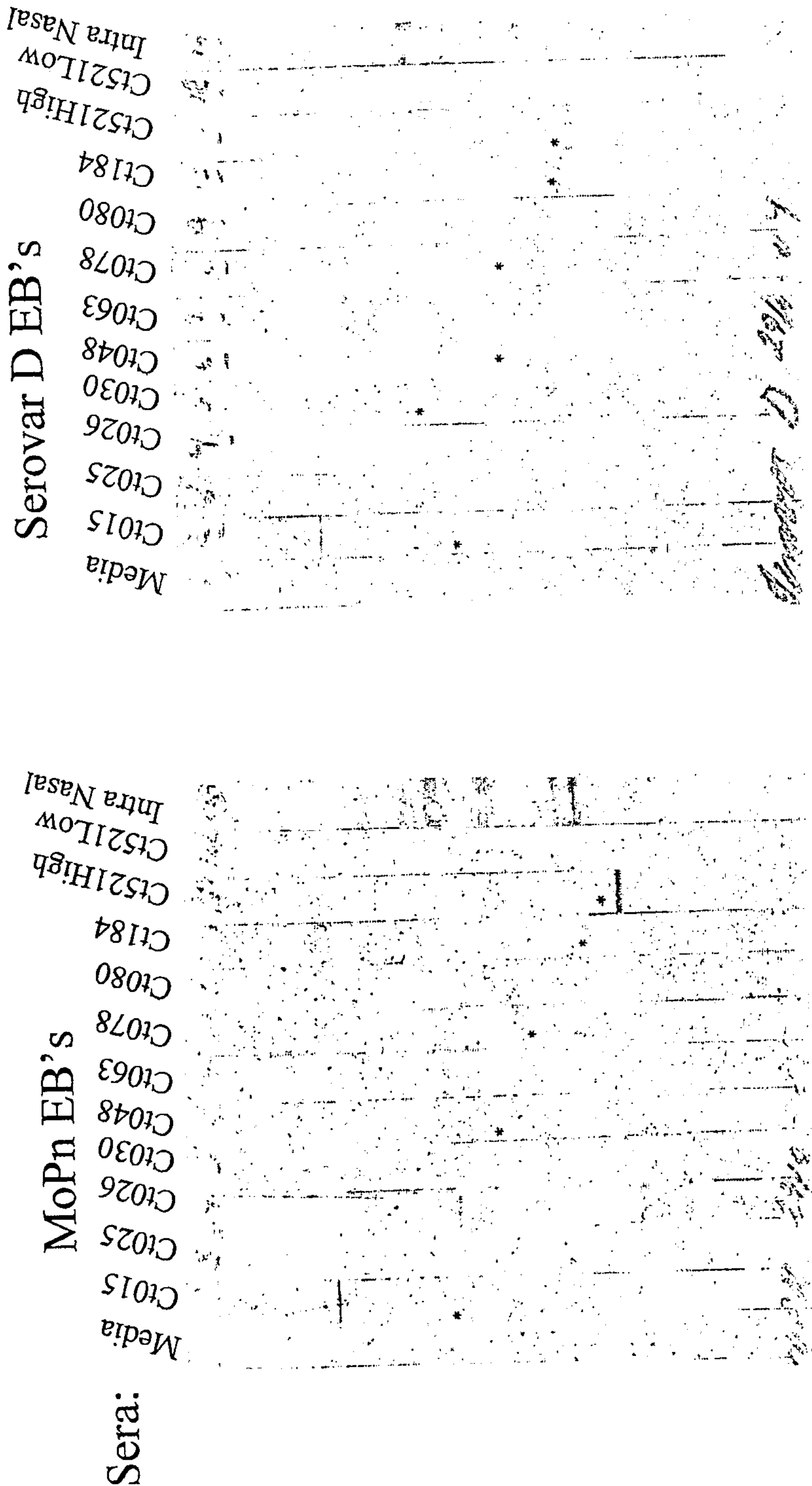


Fig. 14



Serum reactivity against whole Chlamydia EB



- Positive sera:**
- Ct150 Ct184 Ct521High
 - Ct048
 - Ct078
- Positive sera:**
- Ct050 Ct078
 - Ct030 Ct184
 - Ct048 Ct521High

Fig. 15

Fig. 16

IFN γ - 3 wks postvacc - restimulated splenocytes

