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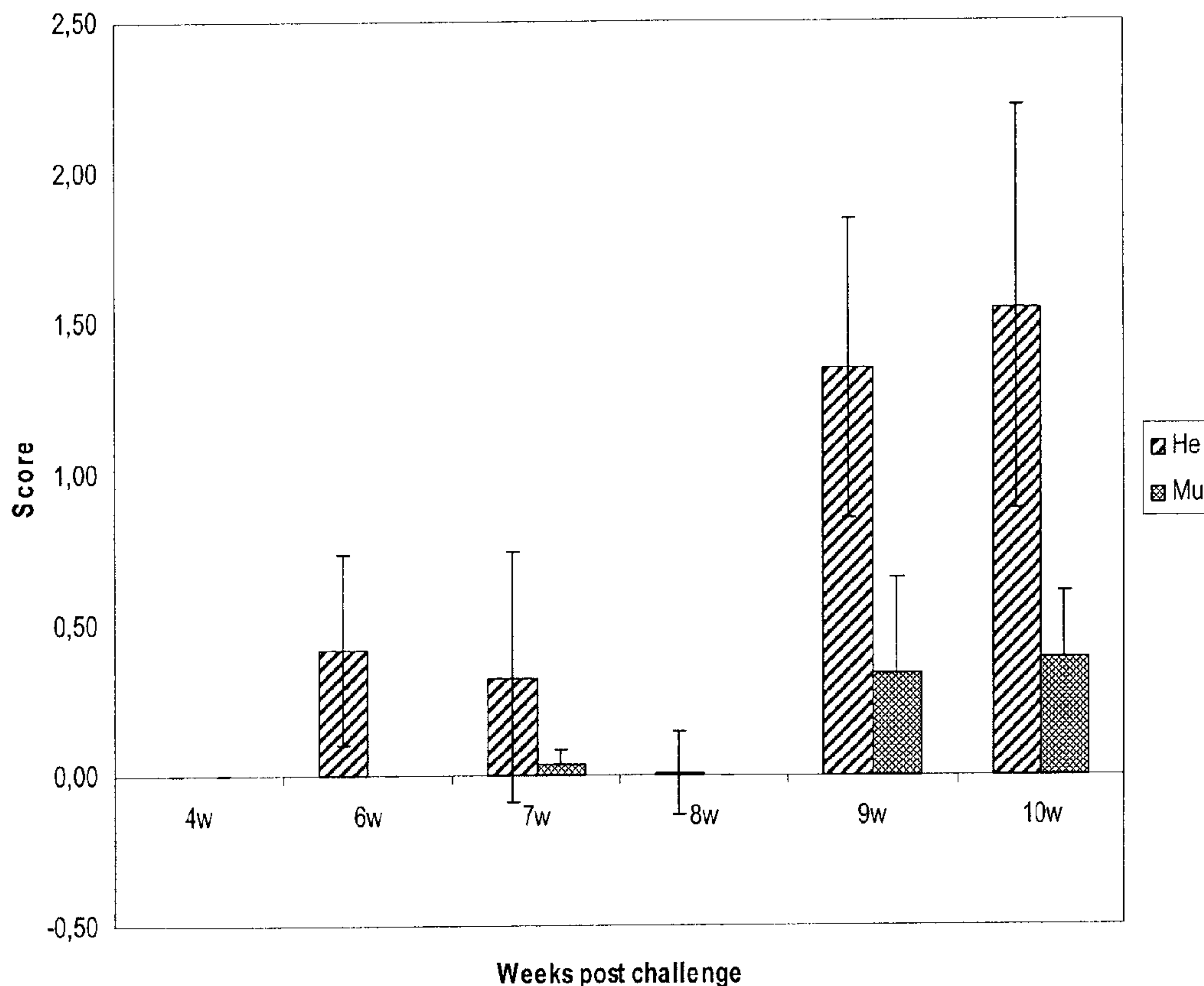
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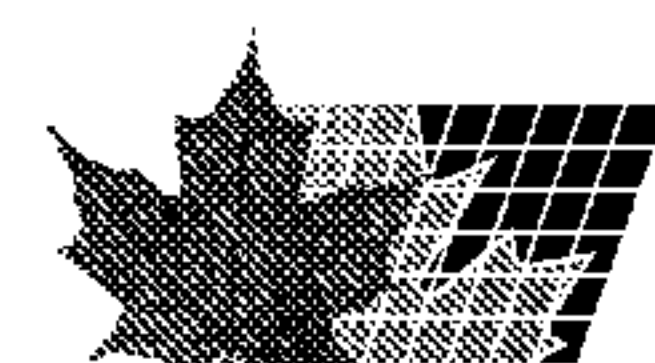
(54) **Titre : AGENT PATHOGENE INEDIT DE POISSON**

(54) **Title: NOVEL FISH PATHOGEN**



(57) **Abrégé/Abstract:**

The present invention provides a fish virus, which when injected into a fish has the ability to introduce the symptoms and disease of Cardiomyopathy Syndrome (CMS). Further, a vaccine is provided comprising a virus according to the invention or a component or part of said virus. The invention further provides a method for isolating or producing the virus. The invention further provides use of the virus for the management of diseases in fish.



Abstract

The present invention provides a fish virus, which when injected into a fish has the ability to introduce the symptoms and disease of Cardiomyopathy Syndrome (CMS). Further, a vaccine is provided comprising a virus according to the invention or a component or part of said virus. The invention further provides a method for isolating or producing the virus. The invention further provides use of the virus for the management of diseases in fish.

NOVEL FISH PATHOGEN

Technical field of the invention

The present invention relates to a novel fish pathogen. In particular the invention
5 provides a fish virus, which when injected intraperitoneally or intramuscularly into fish, has the ability to introduce the symptoms and disease of Cardiomyopathy Syndrome (CMS). Further the invention pertains to vaccines and therapeutics developed on the basis of this pathogen.

10 Background of the invention

Heart and skeletal muscle inflammation (HSMI) is emerging as a significant disease problem and the Norwegian Food Safety Authority has recommended that it should be classified and handled as a group B disease. The infectious nature of HSMI has been indicated, and the first isolation of the virus was previously
15 reported. By electron microscopy virus-like particles of approximately 70 nm were observed. Most outbreaks are reported in fish transferred to seawater 5-9 months earlier. Cardiac lesions are characterized by myocardial degeneration and infiltration of mononuclear cells in both the spongy and compact layers of the ventricle. Lesions are also regularly observed in the red skeletal muscle localized
20 along the lateral line of the fish, sometimes extended to adjacent white fibres.

Cardiomyopathy syndrome (CMS) is a related disease affecting primarily large Atlantic salmon in the second year in seawater close to harvest, and therefore the economic impact is significant. Affected fish may suddenly die without showing signs of disease, or may show symptoms such as abnormal swimming behaviour
25 and anorexia. CMS is diagnosed on the basis of histopathology, showing severe inflammation and degeneration of spongy myocardium in the atrium and ventricle. A possible secondary effect of circulatory disturbance is multifocal liver necrosis which is commonly observed. Whereas the cause of CMS is still unknown, infectious aetiology has been hypothesized and debated, but not yet proven or
30 rejected. Both intracellular inclusions and virus-like particles have been observed, but reports are not consistent as apparent from a review provided in Kongtorp *et al.* 2005: in 1997 Grotmol *et al.* reported the presence of nodavirus-like particles in endothelial cells in the heart of Atlantic salmon diagnosed as suffering from CMS. The particles had a diameter of 25 nm. However, the assumed virus was
35 never isolated, and infection trials were never conducted to confirm the aetiology of the disease. In 2005 Hodneland *et al.* described the isolation and

characterization of a togavirus, salmonid alphavirus 3 (SAV3) from rainbow trout and Atlantic salmon diagnosed with Pancreatic disease or CMS. This Norwegian subtype of the salmonid alphaviruses is the causative agent of pancreatic disease in Norwegian waters but any involvement of this pathogen in the ethiology of CMS has not been confirmed. Most recently, as documented in a report of November 2007, challenge experiments conducted by the Institute of Aquaculture, University of Stirling, Scotland failed to establish whether CMS is in fact infectious: in these experiments only one of 25 Atlantic salmon developed symptoms of CMS when exposed to cell extracts from kidney and heart from a CMS outbreak. The authors discuss that CMS outbreaks in farmed salmon may be a production problem resulting from fish growing too fast (Report prepared for the Scottish Aquaculture Research Forum by Institute for Aquaculture, University of Stirling, 2007).

Consequently, there is a need to first establish whether CMS has viral or bacterial aetiology and, if this is the case, to isolate and characterise the causative agent. Secondly the economical impact which CMS infections may have in the fish farming industry provides the impetus to develop tools for detecting and diagnosing CMS infections and therapeutics for prophylaxis and treatment of CMS infections.

20

Summary of the invention

The present invention is based on the finding that Cardiomyopathy Syndrome has a viral aetiology, and accordingly, an object of the present invention relates to a fish virus, which when infecting a fish causes the symptoms and/or disease and/or histopathological lesions of CMS.

In particular, it is an object of the present invention to provide a virus which may be useful in the prevention of outbreaks of CMS and/or in the treatment of the disease.

Thus, one aspect of the invention relates to a fish virus, which when injected intraperitoneally or intramuscularly in doses of 0.1 – 0.2 ml/fish of an infected GF-1 cell homogenate prepared from 1st passage of the virus, has the ability to introduce the symptoms and disease of Cardiomyopathy Syndrome (CMS) and wherein:

- i) said virus is the strain deposited under the Budapest Treaty with the European Collection of Cell Culture (ECACC), Health Protection Agency, Porton Down, Salisbury, Wiltshire (UK), SP4 0JG UK on the 29 March 2007 under accession number 07032902 or a strain with related genotypic and/or phenotypic characteristics, and/or

- ii) said virus reacts with immune serum from rabbit raised against the strain deposited under ECACC accession number 07032902.

Another aspect of the present invention relates to a cell line comprising the fish virus as defined above.

- 5 Yet another aspect of the present invention is to provide a vaccine comprising a virus as defined above, or a component or part of said virus.

Further aspects of the invention provide a feed comprising a vaccine according to the invention, an antiserum or an isolated antibody which selectively binds to a virus as defined above or to a component or part of said virus.

- 10 In yet a further aspect the invention provides a method of isolating or producing a virus as defined above, said method comprising
 - i) producing a homogenate of a tissue from a fish suffering from/showing the symptoms of Cardiomyopathy Syndrome (CMS);
 - ii) inoculating a cell culture of a suitable cell line;
 - 15 iii) isolating virus particles from said cell and/or from the medium in which the cell line is cultured.

Also the invention provides a virus as defined above or a part of said virus for use in medicine/veterinary medicine.

- 20 In a related aspect the invention pertains to a virus as defined above or a part of said virus for use in prevention of Cardiomyopathy Syndrome (CMS) in fish and/or for reducing the viral load in a fish and/or for reducing the incidence of CMS in a population of fish and/or for treatment of Cardiomyopathy Syndrome (CMS) in fish and/or for reducing the incidence and/or severity of lesions caused by Cardiomyopathy Syndrome Virus in fish.

- 25 A further aspect relates to the use of the virus as defined above or a part of said virus for the manufacture of a medicament for prevention of Cardiomyopathy Syndrome (CMS) in fish and/or for reducing the viral load in a fish and or for reducing the incidence of Cardiomyopathy Syndrome in a population of fish and/or for treatment of Cardiomyopathy Syndrome in fish, and/or for reducing the

30 incidence and/or severity of lesions caused by Cardiomyopathy Syndrome Virus in fish.

- Finally, still further aspects of the invention provide a method for prevention of Cardiomyopathy Syndrome in fish and/or for reducing the viral load in a fish and or for reducing the incidence of Cardiomyopathy Syndrome in a population of fish

35 and/or for treatment of Cardiomyopathy Syndrome in fish, and/or for reducing the incidence and/or severity of lesions caused by Cardiomyopathy Syndrome Virus in

fish, said method comprising administering to the fish a vaccine according to the invention.

Brief description of the figures

5 Figure 1 shows histological changes in the heart (He) and skeletal muscle (Mu) during a challenge study with HSMIV in Atlantic salmon. Histological changes are scored at different time post challenge,

Figure 2 shows histological changes in the heart (He) and skeletal muscle (Mu) over time in cohabitant Atlantic salmon residing in the same tank as the fish
10 injected with HSMIV,

Figure 3 shows histological changes in the heart (He) and skeletal muscle (Mu) during a challenge study with CMSV in Atlantic salmon. Histological changes are scored at different time post challenge,

Figure 4 shows histological changes in the heart (He) and skeletal muscle (Mu)
15 over time in cohabitant Atlantic salmon residing in the same tank as the fish injected with CMS,

Figure 5 shows stained tissue sections of heart from CMS virus infected fish at 6 weeks post infection; A: Ventricle; no or infrequent inflammatory changes are seen in the epicard. B. Ventricle; focal to multi-focal inflammation is seen in the
20 myocard. C and D: Atrium showing focal inflammation. Circles mark inflammatory loci.

Figure 6 shows stained tissue sections of heart from HSMI virus infected fish at 7 weeks post infection; A and B: Ventricle; inflammation in the epicard extends into the compact layer of the ventricle as marked by arrow in (A). C and D: Atrium –
25 no inflammation seen.

Figure 6 C and D: Atrium from HSMI virus infected fish.

Figure 7: Graph illustrating the prevalence of lesions in the atrium after CSMV challenge of fish which have been vaccinated with a CMS vaccine according to the invention, compared with CMSV challenge of PBS controls and fish having received
30 an HSMI vaccine

Figure 8: Graph illustrating the prevalence of lesions in the ventricle after CSMV challenge of fish which have been vaccinated with a CMS vaccine according to the invention, compared with CMSV challenge of PBS controls and fish having received an HSMI vaccine

Figure 9: Electron micrograph of cells infected with virus according to the invention, showing spherical structures having an approximate diameter of 70 nm.

The present invention will now be described in more detail in the following.

5

Detailed description of the invention

Definitions

Prior to discussing the present invention in further details, the following terms and
10 conventions will first be defined:

The term "genotypic characteristics" refers broadly to the composition of one or more parts of an individual's genome which contributes to determining a specific trait of the individual. In the context of the present invention, genomic characteristics of the virus may be assessed by RT-PCR using species specific
15 primers.

The term "phenotypic characteristics" refers equally broadly to one or more observable properties of an organism that are produced by the inherited genotype or by interaction of the inherited genotype of the individual with transmitted epigenetic factors, and/or non-hereditary environmental variation or factors. In
20 the context of the present invention, the term "related phenotypic characteristics" includes any of the following characteristics: size, shape, density, pH stability, temperature stability, chloroform sensitivity and haemagglutination.

"Phenotypic characteristics" further include the ability to induce symptoms or clinical signs of CMS as described in the present application. "Phenotypic
25 characteristics" also include the ability of the virus to introduce such clinical signs in a laboratory challenge experiment when a homogenate from a cell culture or a tissue infected with the CMS virus is injected in Atlantic Salmon (*Salmo Salar* L.) as described herein.

The term "essentially free of other viral or microbial material" as used herein
30 refers to a preparation of a virus according to the invention, wherein the virus has been removed from its natural genetic milieu, and is thus largely free of other extraneous or unwanted viral or microbial material. Thus, when substantially free of other viral or microbial material, the virus of the invention is in a preparation containing at the most 10% by titre or volume of other viral or microbial material
35 (lower percentages of other viral or microbial material are preferred, e.g. at the most 8% by titre or volume, at the most 6% by titre or volume, at the most 5% by titre or volume, at the most 4% by titre or volume, at the most 3% by titre or

volume, at the most 2% by titre or volume, at the most 1% by titre or volume, at the most 0.5% by titre or volume, at the most 0.1% by titre or volume, or at the most 0.05% by titre or volume).

The term "substantially free of other viral or microbial material" as used herein
 5 refers to a preparation of a virus according to the invention, wherein the virus is in a preparation in which other viral or microbial material cannot be detected using conventional techniques like seeding on TSA or cystein heart agar spread plates, seeding in cell cultures known to support the growth of known fish viruses like Pancreas Disease Virus, Infectious Salmon Anemia virus and Infectious
 10 pancreatic Necrosis virus and PCR with primers designed against known sequences from fish pathogens. Further, it is to be understood that when "substantially free of other viral or microbial material" the virus of the invention is in a form wherein it may be used for therapeutic purposes.

It is to be understood that providing virus according to the invention in
 15 preparations being essentially or substantially free of other viral or microbial material does not exclude the possibility of combining such preparations with other viral or microbial material, such as in the polyvalent vaccines disclosed herein.

The term "component or part of said virus" refers to a component or part of the
 20 nucleic acid core of the virus or of the surrounding protein coat. In the context of the present invention, it is preferred that the said component or part possesses antigenicity. Antigenicity may imply a capacity of the component or part of said virus to induce an immune response, that is to be recognized by and interact with an immunologically specific antibody or T-cell receptor; or a capacity to produce
 25 immunity.

Aspects and embodiments of the invention

Virus

30 A first aspect of the present invention pertains to a virus which is the causative agent of Cardiomyopathy Syndrome. In particular the invention provides a fish virus, which when injected intraperitoneally or intramuscularly in doses of 0.1 – 0.2 ml/fish of an infected GF-1 cell homogenate prepared from 1st passage of the virus, has the ability to introduce the symptoms and disease of Cardiomyopathy
 35 Syndrome (CMS) and wherein:

- i) said virus is the strain deposited under the Budapest Treaty with the European Collection of Cell Culture (ECACC), Health Protection

Agency, Porton Down, Salisbury, Wiltshire (UK), SP4 0JG UK on the 29 March 2007 under the accession number 07032902 or a strain with related genotypic and/or phenotypic characteristics, and/or

- ii) said virus reacts with immune serum from rabbit raised against the deposited strain under ECACC accession number 07032902.

Particular embodiments of the invention pertain to virus being obtainable from said deposited isolates.. As the skilled person will realize, replication of viral genomes may be accompanied by very high mutation rates. In RNA viruses this is due to the lack of proofreading activity of RNA virus polymerases, which leads to a constant generation of new genetic variants e.g. during virus propagation. Also, it is known that different constellations of mutations may be associated with a similar biological behaviour of the virus. Accordingly, the invention includes viruses which are obtainable from the strain deposited under the Budapest Treaty with the European Collection of Cell Culture (ECACC), Health Protection Agency, Porton Down, Salisbury, Wiltshire (UK), SP4 0JG UK on the 29 March 2007 under accession number 07032909, such as by passage of the virus on a cell line or by genetic manipulation. As illustrated in the present application, a useful cell line for the purpose of culturing the virus according to the invention is a GF-1 cell line; GF-1 cells are described in US patent 6,436,702, and have been deposited in the ATCC under deposit no. PTA-859. Genetic manipulation comprises for instance introduction of substitutions and/or deletions of single or multiple nucleic acid residues.

In relation to the present invention the ability of the virus to introduce the symptoms and disease of CMS symptoms was demonstrated when a homogenate from a cell culture infected with the CMS virus was injected in Atlantic Salmon (*Salmo Salar* L.). As the skilled person will acknowledge the virus of the invention is likely to cause similar symptoms after having been injected into or after having infected fish from species within the salmonidae family. In particular the virus is likely to cause similar symptoms and disease in fish from the salmo subfamily of the salmonidae.

Specifically, the invention provides a virus in relation to which it is characteristic that said symptoms are seen when the cell homogenate is injected in a dose of 0.1 ml on each side of the fish in the lateral muscle.

Further, the symptoms may be seen in at least 20% of individuals in a population of fish, such as in at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90% or in at least 95% of individuals in a population of fish.

In particular, the symptoms may appear 6 weeks post challenge or later, such as 7 weeks post challenge or later, 8 weeks post challenge or later, 9 weeks post challenge or later or 10 weeks post challenge or later.

In particular embodiments relating to the invention, the virus has the ability of
5 introducing:

- i) Inflammatory changes in areas of the spongy myocardium in the atrium and/or the ventricle; and/or
- ii) Abnormal swimming behaviour and/or anorexia; and/or
- iii) Multifocal liver necrosis.

10 While some variation in the location and severity of the symptoms is to be expected, the inflammatory changes in areas of the spongy myocardium in the atrium and/or the ventricle may appear as foci of degeneration and infiltration of inflammatory cells, including lymphocytes and/or macrophages, and/or as areas of necrosis, said areas optionally extending along fibres, the foci ranging in
15 number from 1->5 per viewed field.

The virus according to the invention may have the ability of introducing one or more symptoms selected from the group consisting of: skin haemorrhages, raised scales, exophthalmos, ascites, fibrinous casts over the liver capsule, blood or blood clots filling the pericardial cavity, ruptures in the cardiac atrial wall, dilation
20 of the cardiac atrium, compression of the cardiac ventricle, inflammation of the epi- and endocardium, liver lesions including multifocal to anastomosing necrosis of hepatocytes and fibrinous coating of the capsule, congestion of the spleen and/or the gills.

When isolated using rate-zonal density gradient centrifugation on a sucrose
25 gradient, the virus according to the invention localized in a fraction having a refractive index between 1,3950 and 1,4000 and a density in between 1,160 g/cm³ and 1,175 g/cm³, and/or in a fraction having a refractive index between 1,365 and 1,75 and a density in between 1,090g/cm³ and 1,110g/cm³.

The fact that the virus according to the invention is localized in two separate
30 fractions is likely due to the fact that the virus adheres to larger particles/cell debris during the process. Therefore, a part of the virus isolate will be located in the top part of the gradient. It is contemplated that the true density of the virus is in the range of 1,16-1,17 g/cm³ when analysed on a sucrose gradient.

For the purpose of analysing the virus according to the invention the rate-zonal
35 density gradient centrifugation was performed using as starting material the supernatant from a culture of GF-1 cells infected with the virus. The supernatant was centrifuged at 17700xg for 30 min, re-suspended in 4 ml culture media and

frozen at -80°C. The supernatant was spun at 100000xg for 3h and the pellet was re-suspended in PBS and frozen at -80°C.

The pellet material was diluted in PBS and loaded on to a sucrose density gradient consisting of: 6 ml 36% sucrose in PBS w/v, 6 ml 42% sucrose in PBS w/v, 6 ml
5 48% sucrose in PBS w/v, 6 ml 54% sucrose in PBS w/v, and 6 ml 60% sucrose in PBS w/v which was subsequently spun at 100000xg at 4°C for 16h. Fractions were collected in 2 ml aliquots from the bottom of the tube, and the refractive index was measured in each fraction.

Initial studies suggested that the virus according to the invention has a diameter
10 of 60-100 nm, such as a diameter of 60-70 nm, a diameter of 65-75 nm, a diameter of 70-80 nm, a diameter of 75-85 nm, a diameter of 80-90 nm, a diameter of 85-95 nm or a diameter of 90-100 nm.

Inspection of electron micrographs (EM) of infected cell cultures suggested, that the virus according to the invention is a spherical virus. Further the EM analyses
15 indicated that the virus according to the invention has an approximate diameter of 70 nm. By "an approximate diameter of 70 nm" is to be understood a diameter in the range of 65-75 nm, such as in the range of 67-75 nm, 67-73 nm, 65-73 nm, or such as 67-73 nm.

Analyses by PCR on reverse transcribed RNA from the virus according to the
20 invention has indicated that the virus has genotypic characteristics which are different from those of the salmonid alphaviruses, infectious pancreatic necrosis virus (IPNV), and nodavirus, including grouper nervous necrosis virus,

In particular, this was shown in studies where 1,7µg RNA isolated from the virus according to the invention was reverse transcribed using random hexamers as
25 primers.

PCR was performed on the reverse transcribed products using the following primers:

Primers specific for salmonid alphavirus:

forward primer - CGTCACTTTCACCGAGCGACTCCCAGACG (SEQ ID NO: 1)

30 reverse primer - GGATCCATTCGGATGTGGCGTTGCTATGG (SEQ ID NO: 2)

Primers specific for IPNV:

Forward primer - GTCCGGTGTAGACATCAAAG (SEQ ID NO: 3)

Reverse primer - TGCAGTTCCTCGTCCATCC (SEQ ID NO: 4);

Primers specific for Grouper nervous necrosis virus (Noda-virus):

35 Forward primer - GGATTTGGACGTGGGACCAA (SEQ ID NO: 5)

Reverse primer - CGGATGACCCGGTTAGTTTTTC (SEQ ID NO: 6);

The analysis is performed using in a standard PCR reaction, comprising heating of the sample to 95°C for 5 min, followed by 30 cycles of denaturing at 95 °C for 30 sec, annealing at 50 °C for 30 sec, and elongation at 72 °C for 60 sec, followed by a single step of heating to 72 °C for 10 min. While a detailed description of the RT-PCR methodology is provided in example 7 in the present application, it will be within the capacity of the skilled person to conduct such analyses using general knowledge on the requirements for buffers, polymerases, primers, nucleotide mixtures and the like.

Using standard PCR conditions and temperatures as described above it was not possible to detect any significant product formation in any of the tests. Accordingly, the CMS virus as provided herein does not contain a ribonucleic acid species capable of forming significant amounts of a product with any one of the PCR primer pairs selected from the group consisting of:

- i) a forward primer having a nucleotide sequence as set forth in SEQ ID NO: 1 and a reverse primer having a nucleotide sequence as set forth in SEQ ID NO: 2;
- ii) a forward primer having a nucleotide sequence as set forth in SEQ ID NO: 3 and a reverse primer having a nucleotide sequence as set forth in SEQ ID NO: 4; and
- iii) a forward primer having a nucleotide sequence as set forth in SEQ ID NO: 5 and a reverse primer having a nucleotide sequence as set forth in SEQ ID NO: 6;

when analysed in a standard PCR reaction on reverse transcribed RNA isolated from the fish virus, comprising heating of the sample to 95°C for 5 min, followed by 30 cycles of denaturing at 95 °C for 30 sec, annealing at 50 °C for 30 sec, and elongation at 72 °C for 60 sec, followed by a single step of heating to 72 °C for 10 min. As illustrated in example 6 of the present application, studies have been conducted in order to assess the ability of the virus according to the invention to grow in cultures of various cell lines which are commonly used to support growth of known viral fish pathogens. As demonstrated, the fish virus according to the invention, when provided in a tissue homogenate from an infected fish, is incapable of growing in any cell line from the group consisting of: BF-2 (Bluegill fry, caudal trunk, available from LCG Promochem as ATCC® Number: CCL-91™), BB (Brown bullhead, connective tissue and muscle, available from LCG Promochem as ATCC® Number: CCL-59™), RTG-2 (Rainbow trout, gonadal tissue, available from LCG Promochem as ATCC® Number: CCL-55™), SHK-1 (salmon head, kidney, established by Dannevig et al. as reported in 1995 and commonly available in research laboratories), CHSE-214 (Chinook salmon, embryo, available

from LCG Promochem as ATCC® Number: CRL-1681™), CCO (Channel catfish, ovary, available from LCG Promochem as ATCC® Number: CRL-2772™), CHH-1 (Chum salmon, heart fibroblast, available from LCG Promochem as ATCC® Number: CRL-1680™) and FHM (Fat head minnow, epithelial like, available from
 5 LCG Promochem as ATCC® Number: CCL-42™).

As mentioned in the example most of these cell lines are permissive for one or more known viral fish pathogens, providing further indication that the virus according to the invention has phenotypic characteristics which are different from those of Infectious Pancreatic Necrosis Virus (IPNV), Viral Haemorrhagic
 10 Syndrome Virus (VHSV), Infectious Haematopoietic Necrosis Virus (IHNV), Channel Catfish Virus (CCV), Spring Viremia of Carp (SVCV), Infectious salmon Anemia Virus, Pancreatic Disease Virus (PDV) and Catfish Tumour Virus (CTV).

In a further embodiment the virus according to the invention is inactivated by incubation with 10% v/v chloroform for 30 minutes at 20°C with continuous
 15 shaking. The sensitivity towards chloroform treatment indicates the presence of an envelope containing essential lipids.

In yet a further embodiment the fish virus according to the invention is inactivated by incubation at 70°C for 30 minutes while it is insensitive to incubation at 60°C for 30 minutes.

20 In other embodiments the culture of the fish virus according to the invention is insensitive to incubation with 100 µg/ml DNA synthesis inhibitor bromodeoxyuridine (BrdU). In these embodiments the insensitivity to DNA synthesis inhibitors indicates that the virus according to the invention is an RNA virus.

25 In still other embodiments the fish virus according to the invention is inactivated after incubation 4 hours with pH 3.0 at 15°C, whereas pH 5.0 has no effect compared with pH 7.2.

It is within the scope of the present invention to provide an isolate of the virus described above. The virus of the invention may thus be in an isolated form
 30 and/or substantially or essentially free of other viral or microbial material.

It is further within the scope of the invention to provide a virus as described above which is either attenuated or inactivated. Several approaches for obtaining attenuated virus are available to the skilled person. An attenuated strain of a virus may be generated for instance by passing the virus through cell culture a number
 35 of times, or deleting or mutating a gene involved in its replication pathway.

Inactivation of the virus may be obtained by chemical or physical means. Chemical inactivation can be carried out by treatment of the viruses by for

example, but not limited to, treatment with enzymes, with formaldehyde, β -propiolactone or ethyleneimine or a derivative thereof, with organic solvent (e.g. halogenated hydrocarbon) and/or detergent, e.g. Triton® or Tween®. Physiological inactivation can advantageously be carried out by subjecting the viruses to
 5 energy-rich radiation, such as UV light, gamma irradiation or X-rays. If necessary, the inactivating agent can be neutralized with thiosulphate. If required, the pH is subsequently returned to about pH 7.

Infected cell line

10 A second aspect of the invention provides a cell line comprising the fish virus as defined in above. Several cell lines may be used for propagation of the virus. In preferred embodiments, however, the cell line is derived from *Epinephelus coioides*.

In particular embodiments the cell line is a GF-1 cell line; GF-1 cells are described
 15 in US patent 6,436,702, and have been deposited in the ATCC under deposit no. PTA-859. Another example of a useful cell line is the ASL cell line derived from Atlantic salmon liver. Cells were isolated from Atlantic salmon (*Salmo salar*) liver by a two-step collagenase perfusion procedure according to Dannevig and Berg (1985). The cells have been sub-cultured more than 20 times.

20

Vaccine

Another aspect of the invention pertains to a vaccine comprising a virus as defined above, or a component or part of said virus.

The skilled person will realise that the content of a vaccine preparation varies
 25 depending on the intended use of the vaccine. In presently preferred embodiments of the invention, such as the test vaccine which is used in the clinical trial illustrated in Example 10 herein very low amounts of antigen are used. As illustrated in the example, good protection against histopathological changes typical for CMS are shown in fish vaccinated with CMS vaccine containing
 30 for instance 0.016 mg antigen/dosage or 0.16 mg antigen/ml of vaccine formulation. According to these embodiments the vaccine comprises an amount of antigen, which is in the range of 0.05-1.0 mg/ml, such as from 0.15 to 0.5 mg/ml, from 0.15 – 0,4 mg/ml, from 0.15 – 0,3 mg/ml or such as from 0.1 to 0.5 mg/ml, from 0.1 – 0,4 mg/ml, from 0.1 – 0,3 mg/ml, from 0.1 – 0,25 mg/ml or
 35 such as from 0.1 – 0.2 mg/ml. The vaccine may be for administration in dosages

of 0.005-0.5 mg/individual, preferably from 0.01-0.05 mg/individual, or such as from 0.01-0.02 mg/individual.

In a final product approved for commercial use in aquaculture, the preferred amount of antigen may be somewhat higher. According to embodiments

5 pertaining to such products the preferred amount of antigen may be in the range of 0.5-2.0 mg/ml, such as from 0.5 to 1.5 mg/ml, from 0.5 – 1,3 mg/ml, from 0.6 – 1,5 mg/ml or 0.7-1.5 mg/ml, such as from 0.8 to 1.5 mg/ml, from 0.9 – 1,5 mg/ml, from 0.7 – 1,4 mg/ml, from 0.7-1.3 mg/ml, from 0.7-1.2 mg/ml, such as from 0.8 to 1.4 mg/ml, from 0.8 – 1,3 mg/ml, from 0.8 – 1,2 mg/ml or from 0.9-
10 1.4 mg/ml, such as from 0.9 to 1.3 mg/ml, from 0.9 – 1,2 mg/ml. For such embodiments a most preferred amount of antigen of 1 mg/ml is presently contemplated. Additional embodiments provide a vaccine comprising an amount of antigen corresponding to a TCID₅₀ of 10⁵-10¹⁰ per dosage, such as an amount of antigen corresponding to a TCID₅₀ of 10⁶-10⁹ per dosage, an amount of antigen
15 corresponding to a TCID₅₀ of 10⁷-10⁹ per dosage, such as an amount of antigen corresponding to a TCID₅₀ of 5x10⁷-5x10⁸ per dosage, such as an amount of antigen corresponding to a TCID₅₀ of 8x10⁷-4x10⁸ per dosage, such as an amount of antigen corresponding to a TCID₅₀ of 1x10⁸-4x10⁸ per dosage, such as an amount of antigen corresponding to a TCID₅₀ of 1x10⁸-3x10⁸ per dosage, such as
20 an amount of antigen corresponding to a TCID₅₀ of 1-2x10⁸ or such as an amount of antigen corresponding to a TCID₅₀ of 1.5x10⁸ per dosage.

The vaccine may be in the form of a suspension of the virus or it may be lyophilized. In a lyophilized vaccine it may be useful to add one or more stabilizers. Suitable stabilizers are for example carbohydrates such as sorbitol,
25 mannitol, starch, sucrose, dextran; protein containing agents such as bovine serum or skimmed milk; and buffers such as alkali metal phosphates.

The vaccine according to the invention may further be in a formulation comprising an adjuvant. Examples of adjuvants frequently used in fish and shellfish farming are muramyl dipeptides, lipopolysaccharides, several glucans and glycans, mineral
30 oil, Montanide™ and Carbopol®. An extensive overview of adjuvants suitable for fish and shellfish vaccines is given in the review paper by Jan Raa (1996).

The vaccine of the invention may further comprise a suitable pharmaceutical carrier. In a currently preferred embodiment the vaccine is formulated as an emulsion of water in oil. The vaccine may also comprise a so-called "vehicle". A
35 vehicle is a device to which the antigen adheres, without being covalently bound to it. Such vehicles are i.a. biodegradable nano/micro-particles or -capsules of PLGA (poly-lactide-co-glycolic acid), alginate or chitosan, liposomes, niosomes, micelles, multiple emulsions and macrosols, all known in the art. A special form of

such a vehicle, in which the antigen is partially embedded in the vehicle, is the so-called ISCOM (European patents EP 109.942, EP 180.564 and EP 242.380).

In addition, the vaccine may comprise one or more suitable surface-active compounds or emulsifiers, e.g. Cremophore®, Tween® and Span®. Also
5 adjuvants such as interleukin, CpG and glycoproteins may be used.

It is to be understood that the vaccine may further be in a formulation comprising an antigen from a bacterial source, an antigenic material obtained from a viral source other than the fish virus as defined above, an antigenic material obtained from a parasitical source, and/or an antigenic material obtained from a fungal
10 source. Polyvalent vaccines containing antigens from typical fish pathogens other than CMS virus are well known in the art and are already commercially available. In addition, representative isolates of relevant fish pathogens are available from various sources.

In particular embodiments of the invention said antigen from a bacterial source is
15 selected from the group consisting of: live, attenuated or killed bacteria of the species *Piscirickettsias* sp., *Aeromonas* sp., *Vibrio* sp., *Listonella* sp., *Moritella viscosa*, *Photobacterium damsela*, *Flavobacterium* sp., *Yersinia* sp., *Renibacterium* sp., *Streptococcus* sp., *Lactococcus* sp., *Leuconostoc* sp., *Bifidobacterium* sp., *Pediococcus* sp., *Brevibacterium* sp., *Edwardsiella* sp., *Francisella* sp.,
20 *Pseudomonas* sp., *Cytophaga* sp., *Nocardia* sp., *Mycobacterium* sp., parts or subunits of these bacteria, and any combination hereof.

Isolates of such bacteria are available, e.g. from LGC Promochem/American Type Culture Collection ATCC repository and distribution center (ATCC) including strains of *A. salmonicida* (ATCC 33658), *V. salmonicida* (ATCC 43839), *V. anguillarum*
25 serotype O1(ATCC 43305) and O2(ATCC 19264), and *Moritella viscosa* (ATCC BAA-105). In addition, cultures of *Piscirickettsias salmonis* have been deposited in the European Collection of Cell Culture (ECACC), Health Protection Agency, Porton Down, Salisbury, Wiltshire (UK), SP4 0JG UK on the 9 June 2006 under the following accession numbers: 06050901, 06050902, 06050903 and 07032110.

Other specific embodiments pertain to a vaccine, wherein said antigenic material
30 obtained from a viral source other than the fish virus as defined above is from a virus selected from the group consisting of: Viral Hemorrhagic Septicemia Virus (VHSV), Viral Hemorrhagic Septicemia Virus (VHSV), Infectious Hematopoietic Necrosis virus (IHNV), Infectious Pancreatic Necrosis Virus (IPNV), Spring Viremia
35 of Carp (SVC), Channel Catfish Virus (CCV), Infectious Salmon Anaemia virus (ISAV), pancreatic disease virus (SPDV), Iridovirus, and heart and skeletal muscle inflammation virus (HSMIV), parts or subunits of any one of these viruses, and

combinations hereof. Representative species of such viruses are available to the skilled artisan, for instance from the following deposits: infectious pancreatic necrosis virus (IPNV, ATCC VR-1318, country of origin: unknown), Viral Hemorrhagic Septicemia Virus (VHSV, ATCC VR-1389, country of origin:

5 Denmark); Infectious Hematopoietic Necrosis virus (IHNV, ATCC VR-1392, country of origin: USA)); Pancreatic Necrosis Virus; Spring Viremia of Carp (SVC, ATCC VR-1390, country of origin: Denmark); Channel Catfish Virus (CCV) (ATCC VR-665, country of origin: USA); Infectious Salmon Anaemia (ISA) virus (ATCC VR-1554, country of origin: Canada).

10 Patent deposits have previously been made by the present applicant of the following viral species: Heart and Skeletal Muscle Infection Virus (HSMIV, patent deposit nr ECACC 04050401, country of origin: Norway).

In more specific embodiments, said antigenic material obtained from a viral source other than the fish virus as defined above is from the group consisting of:

15 Glycoprotein of Viral Hemorrhagic Septicemia Virus (VHSV), nucleoprotein of Viral Hemorrhagic Septicemia Virus (VHSV), glycoprotein of Infectious Hematopoietic Necrosis virus (IHNV), nucleoprotein structural proteins of Infectious Pancreatic Necrosis Virus (IPNV), G protein of Spring Viremia of Carp (SVC), and a membrane-associated protein, tegumin or capsid protein or glycoprotein, of
20 Channel Catfish Virus (CCV), antigenic fragments of any of one of these proteins and combinations hereof.

In other embodiments said antigenic material from a parasitic source is from a source selected from the group consisting of *Lepeophtheirus Sp.*, *Caligus Sp.*, and *Ichthyophthirius Sp.*, parts of any one of these parasites, and combinations hereof.

25 In yet other embodiments said antigenic material is from a fungal source selected from the group consisting of *Saprolegnia Sp.*, *Branchiomyces sanguinis*, *Branchiomyces demigrans* and *Ichthyophonus hoferi*.

The vaccine according to the invention may in particular be formulated for administration to a fin fish. More specifically the vaccine may be (formulated) for
30 administration to a teleostei. The teleostei include, but are not limited to salmonids, basses, breams, cods, snappers, flatfish, catfish, yellowtails and tilapias.

In a presently preferred embodiment the vaccine is formulated for administration to Atlantic salmon (*Salmo Salar L.*), Rainbow trout (*Oncorhynchus mykiss*) and/or
35 Coho salmon (*Oncorhynchus kisutch*)

In further embodiments of the invention the vaccine is formulated for administration by a route selected from the group consisting of: Bath, immersion, intraperitoneal injection, intramuscular injection and oral administration.

Optionally, the vaccine would be administered to young fish in the fresh-water stage.

Feed comprising vaccine

- 5 In a further aspect, the present invention provides a feed comprising the vaccine according to the invention, said feed may for example be pelleted or extruded feed.

Antiserum/antibody

- 10 Another aspect of the invention provides an antiserum or an isolated antibody which selectively binds to a virus as defined above or to a component or part of said virus. Antisera are conventionally obtained for instance by immunising a laboratory animal with the appropriate antigenic determinant. Once the concentration of antibodies in serum from the animal reaches a desired level, the
15 animal is bled. The Immune serum obtained should contain antibodies produced in response to the immunogenic stimulus.

Likewise, techniques for the preparation of antibodies are known to the skilled person. The techniques include the traditional hybridoma technology and alternative techniques such as mRNA display, ribosome display, phage display and
20 covalent display.

In some embodiments of the invention the isolated antibody comprises a marker, e.g. a radiolabel, a fluorescent tag, a chemiluminescent label or an enzyme. The antibody may be a polyclonal or monoclonal antibody.

In the context of the present invention immune sera and/or isolated antibodies
25 raised against the deposited virus strain provided according to the invention will be useful in identifying virus which are identical to the deposited strain or which are obtainable from said strain. Also, the immune sera and/or isolated antibodies will be effective in identifying virus having phenotypic or genotypic strains which are related to those of the said deposited strain.

30

Method of isolating virus

Another aspect of the invention provides a method of isolating or producing a virus as defined above, said method comprising

- 35 iv) producing a homogenate of a tissue from a fish suffering from/showing the symptoms of Cardiomyopathy Syndrome (CMS);
v) inoculating a cell of a suitable cell strain;

- vi) isolating virus particles from said cell and/or from the medium in which the cell is cultured.

The virus according to the invention may be grown on a cell culture such as the as culture of GF-1 cells. The viruses thus grown may be harvested by collecting the
5 cell culture fluids and/or cells. The virus yield can be promoted by techniques that improve liberation of virus from the grown substrate, e.g. sonication.

Medical use

An additional aspect of the invention provides a virus as defined above or a part of
10 said virus for use in medicine/veterinary medicine.

Provided in a further aspect of the invention is a virus as defined above or a part of said virus for use in prevention of Cardiomyopathy Syndrome (CMS) in fish and/or for reducing the viral load in a fish and or for reducing the incidence of CMS in a population of fish and/or for treatment of Cardiomyopathy Syndrome
15 (CMS) in fish and/or for reducing the incidence and/or severity of lesions caused by Cardiomyopathy Syndrome Virus in fish.

Provided in yet a further aspect is use of the virus as defined above or parts of said virus for the manufacture of a medicament for prevention of Cardiomyopathy Syndrome (CMS) in fish and/or for reducing the viral load in a fish and or for
20 reducing the incidence of Cardiomyopathy Syndrome in a population of fish and/or for treatment of Cardiomyopathy Syndrome in fish and/or for reducing the incidence and/or severity of lesions caused by Cardiomyopathy Syndrome Virus in fish.

25 Preventive/therapeutic method

Also provided in relation to the present invention is a method for prevention of Cardiomyopathy Syndrome in fish and/or for reducing the viral load in a fish and or for reducing the incidence of Cardiomyopathy Syndrome in a population of fish and/or for treatment of Cardiomyopathy Syndrome in fish and/or for reducing the
30 incidence and/or severity of lesions caused by Cardiomyopathy Syndrome Virus in fish, said method comprising administering to the fish a vaccine as described in details above.

Throughout the present specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated
35 element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

With respect to the above description of the various aspects of the present invention and of the specific embodiments of these aspects it should be understood that any feature and characteristic described or mentioned above in connection with one aspect and/or one embodiment of an aspect of the invention
5 also apply by analogy to any or all other aspects and/or embodiments of the invention described.

All patent and non-patent references cited in the present application, are hereby incorporated by reference in their entirety.

The invention will now be described in further details in the following non-limiting
10 examples.

Examples

Example 1: Challenge study comparing HSMI and CMS

15

Heart tissue from Atlantic salmon experiencing a clinical outbreak of HSMI was collected and homogenized with quarts sand in a porcelain mortar, centrifuged at 3000 xg, the supernatant passed through a 45mm syringe filter ((sartorius 17829) and inoculated into GF-1 cell cultures. After 7-10 days, cytoplasmic
20 vacuoles were observed in infected cells. Lysed and filtrated material from these infected cells was further used to infect fresh cell cultures and similar vacuolization was observed.

Inflamed heart tissue was collected from a clinical outbreak of CMS and treated as described above. Clarified heart tissue homogenate was inoculated onto GF-1 cell
25 cultures resulting in cytoplasmic vacuoles in infected cells, with morphology similar to that of HSMI infected cells. Lysed and filtrated material from these infected cells was further used to infect fresh cell cultures and similar vacuolization was observed.

As both diseases are usually observed in seawater, the clinical trial was conducted
30 in seawater. A challenge study was conducted using a "CMS viral isolate" material.

The lysed and filtered material described above was used for intramuscular injection of fish in seawater. Samples of heart and red muscle tissue were collected at defined intervals to follow the development of the disease. The intention was to examine whether material cultured from fish with a CMS or HSMI
35 diagnosis would produce similar or different clinical signs in Atlantic salmon. This

would provide information as to whether CMS and HSMI may be caused by the same or different viral agents.

Objectives

The main objective of this pilot challenge study was to gain knowledge of the development of HSMI and CMS in seawater, and to compare these disease entities in terms of development of histopathological changes in internal organs.

Schedule

10 The major dates were as follows:

Task	Activity	Experimental week
Start of experiment	Transfer of fish to experimental cell	Week -6
Seawater transfer	Transfer of fish to seawater	Week 0
Challenge and control sampling	Tank 1: HSMIV challenge by i.m. injection of 50 fish + 20 cohabitants. Tank 2: "CMSV" challenge by i.m. injection of 50 fish + 20 cohabitants.	Week 0
Sampling	Sampling of 10 control fish Blood and tissue sampling of 6-8 fish at indicated time points from both tanks.	Week 2,4,6,7,8,9,10
End of experiment	End of experiment	Week 10

Materials and methods

Challenge material

15 The challenge isolates were:

- HSMIV 1st passage stock of ALV-702/03, 06.04.04 grown in GF-1 cells, and
- CMSV 1st passage stock from HSMI 1002.06 grown in GF-1 cells.

Environment:

- 5 The fish were smoltified in one tank at 15°C with a 24 hour daylight regime. After transfer to seawater the fish were held at 12 °C.

Challenge

Tank	groups	tagging	<i>challenge isolate/ amount added</i>
1	50 fish i.m. injected	none	<i>HSMIV-1 1st passage stock of ALV-702/03 0.1 ml injected on each side of the fish in the lateral muscle tissue beneath the dorsal fin</i>
	20 cohabitant fish	Adipose fin	<i>none</i>
2	50 fish i.m. injected	none	<i>HSMIV-2 ("CMSV")1st passage stock 0.1 ml injected on each side of the fish in the lateral muscle tissue beneath the dorsal fin</i>
	20 cohabitant fish	Adipose fin	<i>none</i>

10

Intramuscular (i.m.) injection challenge: The fish were anesthetized and 0.1 ml was injected on each side (0.2ml in total) of the fish in the lateral muscle tissue beneath the dorsal fin. Cohabitant fish were marked by clipping the adipose fin and the cohabitants are transferred to the tanks on day of challenge, 20 fish in

15 each of the tanks.

Sampling

Week	33	35	37	39	40	41	42	43
Weeks after challenge	0	2	4	6	7	8	9	10
Challenged fish HSMIV-1 (tank 1)	-	6	6	6	8	8	8	7
Cohabitants tank 1	-	-	-	6	-	6	-	8
Challenged fish HSMIV-2 (tank 2)	-	6	6	6	8	8	8	7
Cohabitants tank 2	-	-	-	6	-	6	-	8
<i>Unchallenged control fish</i>	<i>10</i>	-	-	-	-	-	-	-

Samples

- 5 The samples (# given above) for histological examination were collected from all fish of each time point and treated as given below.

Sample	Sampled in	Post-fixation treatment
<i>Heart tissue</i> <i>Red muscle</i> <i>Liver</i>	<i>Formalin-filled</i> <i>containers</i>	<i>Processed for embedding by</i> <i>standard dehydration</i> <i>methods, embedded in</i> <i>paraffin, sectioned and</i> <i>stained with hematoxylin and</i> <i>eosin. Examination was</i> <i>carried out using a Zeiss light</i> <i>microscope.</i>

Assessment criteria

10

The assessment of histological changes in HSMI challenged fish was performed on the basis of the criteria given below and the scoring was marked on a visual analog scale (VAS).

Inflammation score for HSMI infected fish	Pathological description
0	No pathological changes observed.
1	Mild pathological changes characterized by a limited number (countable) of mononuclear inflammatory cells infiltrating the epicardium, not extending into the compact layer of the ventricle. The infiltration of cells is multifocal to diffuse and can involve parts of or the entire epicardium available for assessment.
2	Moderate pathological changes consisting of high number (uncountable) of inflammatory cells in the epicardium and extending into the compact layer of the heart. The changes in the compact layer can be multifocal or diffuse and typically orient along small blood vessels. A few focal changes can also be seen in the spongy layer.
3	Severe pathological changes characterized by intense infiltration of inflammatory cells in the epicardium, extending into the compact layer, typically with a diffuse distribution pattern and involving the spongy layer in a multifocal pattern. Degeneration and or necrosis of muscle fibers are seen. Atrium can also be involved with inflammatory changes comparable to what is seen in the ventricle.

The criteria listed above were of no use for the evaluation of the changes seen in CMS infected fish. A new score scheme was prepared and the scoring and grading are given below.

Inflammation score for CMS challenged fish	Pathological description
0	No pathological changes observed.
1	Inflammatory changes are seen in the atrium primarily and these appear as foci of degeneration and infiltration of inflammatory cells. The foci are limited in number, 2-3, limited in size and involve a few muscle fibres. A few foci, similar to what is seen in the atrium, can also be observed in the ventricle, typically in the spongy part.
2	Inflammatory changes are seen in the atrium and in the ventricle and these appear as multiple foci of degeneration and infiltration of inflammatory cells. The foci are more numerous (>5 in the atrium and > 3 in the ventricle), involve larger areas of the atrium and the ventricle and several muscle fibres are involved in the changes observed.
3	Inflammatory changes are seen in the atrium and in the ventricle and these appear at this stage as multiple larger foci of degeneration and necrosis, accompanied with infiltration of inflammatory cells, typically macrophages. The foci are several in the atrium, in areas confluent and larger areas of degeneration/necrosis are found in the ventricle, and extend along fibres and can involve several fibres.

Results

HSMI challenged fish

5

The first histological changes typical of HSMI were seen by week 6 in injected fish (4 of 6 examined) with an average score of 0.25 ($\pm 95\%$ conf limits of 0.32) indicating variation between fish. There was no increase seen by 7w and a slight

decline by week 8. A marked increase in number of fish showing changes were found by week 9 and the average score increased to 1,1 ($\pm 95\%$ conf limits of 0,5). The same average score was found by week 10.

- 5 Changes were also found in the red muscle, in 2 fish by week 7, no fish at 8w, 5 fish by week 9 and in 6 fish (of 7 sampled) at 10w. The changes in the red muscle tissue were mild, characterized by infiltration of inflammatory cells, typically around small vessel and in the intermuscular tissue accompanied by a few degenerate muscle cells.

10

The findings are summarized in figure 1 of the present application.

- Histological changes were observed in the cohabitant group (HSMI) by week 10, and the average score in this group was 1,26 ($\pm 95\%$ conf limits of 0,32). One fish
15 was also found with minor changes in the heart by week 8, but the remaining 5 fish at this time point were scored negative. The summary of the findings in the cohabitants at 6, 8 and 10 weeks (when sampling was performed) is given in figure 2.

- 20 It is interesting to note that the average score for the cohabitant fish are as high as in the injected ones and fish also exhibited typical changes in red muscle tissue, 1 of 6 by 6 weeks, and 3 of 8 by 10 weeks.

25 *CMS challenged fish*

- The histological changes seen in the heart of fish challenged with CMS inoculum deviated markedly from the HSMI infected fish. The scoring scheme developed for the HSMI fish was of no use to assess the changes observed in this group and for
30 this reason a new scoring scheme was established.

- The first indications of pathological changes in the CMS group were seen at 6weeks post challenge, where 4 of 6 fish exhibited varying degree of histomorphological changes. In 4 of the fish, the atrium was included in the specimens provided for assessment. By 7 weeks, all fish (8/8) had changes in the
35 heart, and in only 2 fish the atrium was available for evaluation, meaning that the changes observed were found also in the ventricle. At 8 weeks, 8/8 fish were positive (4 of 8 came with atrium present in the sections). By 9 and 10 weeks, all fish showed morphological changes, 4 of 4 had atrium present at 9 weeks, while 5 of 7 had atrium present at 10weeks. The findings are summarized in figure 3 and
40 below.

Note that the skeletal muscle is not included in the figures as no fish infected with the CMS inoculum were found with changes in this organ, at any of the time points examined.

Fish were found positive for typical CMS changes in the cohabitant group by 6 weeks, however with very low scores (2 of 6 fish were positive; 3 of 6 with atrium present in sections). By 8 weeks, 1 fish was found positive and by 10 weeks, 3 of 7 were found positive, at both time points the changes observed were minor (below 0.5 on the visual analogue scale).

Representative histological sections of heart tissue from individuals infected with CMS and HSMI are presented in figures 5 and 6, respectively. As illustrated in figure 5, CMS infection primarily involves the atrium recognised as focal inflammation with associated degeneration and necrosis of muscle cells. Focal inflammation is also seen in the ventricle with degeneration and necrosis of muscle cells and accompanying inflammatory changes. At later stages the inflammatory changes also involve macrophages.

At early time points post infection HSMI infection (figure 6) involves inflammatory changes (lymphocytic) in the epicard. Changes are extending along vessels of the compact layer to involve larger parts over time (diffuse inflammation) while changes in the atrium are infrequently seen.

The findings are summarised in figure 4 and in the comments/summary below.

Comments/summary

The findings in the study can be summarised as follows;

1) HSMI- and CMS-challenge result in two different categories/entities of histological changes in the heart tissue:

- a. HSMI changes are initially found in the epicardium of the ventricle, develop in intensity and extend into underlying muscle tissue involving the spongy part of the ventricle and extend also to the atrium at late stages.
- b. CMS changes are initially found in the atrium but can occur simultaneously in the ventricle. The changes seem to develop more rapidly though in the atrium. Epicardial changes (inflammatory changes) are absent or insignificant in CMS challenged fish.
- c. The characteristics of the inflammatory cells involved in the changes observed are also different, typically lymphocytic in HSMI and

involving more macrophage-like cells in CMS, particularly at late stages of disease.

- 2) The HSMIV and CMSV spread to other fish (cohabitants) over a 10week period, however the findings in this study are that HSMIV spread more readily by cohabitation.

Example 2: Temperature stability and lipid solvent sensitivity test of HSMI and CMS cell culture supernatants.

Intention:

The aim was to determine the lipid solvent sensitivity and temperature stability of HSMI and CMS cell culture supernatants produced in GF-1 cells.

Materials:

Cell cultures and cell culture medium:

- Cell cultures: 25cm² cell culture flasks with 4.4×10^4 GF-1 cells (p31) per cm².
- Growth medium: L-15 supplemented with 10% FBS, 1% L-glutamine and 0.1% Gentamicin

Virus controls:

- Infectious Pancreas Necrosis Virus (IPNV)
 - Pancreas Disease virus (PD)
 - Relevant cell lines
- Both virus controls were diluted in growth medium to a concentration of 8×10^5 viable virus per ml.

Chemicals: Chloroform MERCK 1.02445.1000, K35964445.

Test materials:

- HSMI supernatant: HSMI 1004.06, ALV-702 1p, 20.09.06.
- CMS supernatant: HSMI 1004.06, "CMS" 1p, 20.09.06.

Methods:

Chloroform sensitivity test

Chloroform was added to the two virus controls, the test materials and control growth medium to a final concentration of 10% (v/v), and shaken for 30 minutes
5 at room temperature (ca 20°C). After centrifugation at 2000 x g for 10 minutes, two cell cultures per sample were inoculated with 150µl supernatant. The cell cultures were incubated at 15°C for 19 days.

Temperature stability

10 400 µl aliquots of virus controls and test materials were heated for 30 minutes at 50, 60 and 70°C and then cooled immediately by inoculation of 150µl sample into two cell cultures per sample. The cell cultures were incubated at 15°C for 19 days. The same positive controls as for the chloroform test were used.

Results:

15 Both HSMI and CMS samples were inactivated with chloroform and 30 min treatment at 70°C, whereas temperatures up to 60°C had no effect on the development of CPE/vacuoles after inoculation of GF-1 cell cultures.
PD virus was inactivated both with chloroform and 30 min treatment at 50°C.
IPNV resisted chloroform and 30 min treatment at temperatures up to 70°C, but
20 the infection seemed to be slightly delayed at the highest temperatures.

Temperature stability and chloroform sensitivity of test material and control virus.

Test article	Result / days after inoculation				
	+ ctr (20°C)	50°C	60°C	70°C	Chloroform
HSMIV	+ 11 days pi	+ 11 days pi	+ 11 days pi	- 19 days pi	- 19 days pi
CMSV	+ 7 days pi	+ 7 days pi	+ 7 days pi	- 19 days pi	- 19 days pi
PD virus	+ 6 days pi	- 19 days pi	- 19 days pi	- 19 days pi	- 19 days pi

IPNV	+ 4 days pi	+ 4 days pi	+ 4 days pi	+ 6 days pi	+ 4 days pi
L-15	N/A	N/A	N/A	N/A	- 19 days pi

Conclusions:

- Both HSMI and CMS samples were inactivated with chloroform and 30 min treatment at 70°C, whereas temperatures up to 60°C had no effect on the development of CPE/vacuoles after inoculation of GF-1 cell cultures.
- For HSMI virus, the results confirm the findings in the previous patent application NO20065765/WO05121325.

Example 3: Sensitivity to DNA synthesis inhibitor

10

The purpose is to examine if the use of DNA synthesis inhibitor bromodeoxyuridine (BrdU) inhibits HSMIV and CMS infections in cell lines (GF-1), and thus indicate whether the viruses are RNA or DNA viruses.

BrdU is a synthetic thymidine analog that gets incorporated into a cell's DNA when the cell is dividing (during the S phase of the cell cycle).

Negative control: IPNV (double stranded RNA virus) infected BF-2 cells.

Positive control: Irido virus (DNA virus) infected BF-2 cells.

No of flasks	Virus	Cells	Temp	BrdU (ug/ml)
2	-	GF-1	15 °C	0
2	-	GF-1	15 °C	100
2	HSMIV	GF-1	15 °C	0
2	HSMIV	GF-1	15 °C	100
2	CMS	GF-1	15 °C	0
2	CMS	GF-1	15 °C	100
2	-	BF-2	15 + 25 °C	0
2	-	BF-2	15 + 25 °C	100
2	IPNV	BF-2	15 °C	0
2	IPNV	BF-2	15 °C	100

2	Irido(RSIV)	BF-2	25 °C	0
2	Irido(RSIV)	BF-2	25 °C	100

GF-1 cells, p30: 5×10^4 cells/cm²

BF-2 cells, p25: 8×10^4 cells/cm²

Both cell lines were grown in L-15 supplemented with 10% FBS, 1% L-glutamine
5 and 0.1% Gentamicin in 25 cm² flasks at 15 °C or 25 °C

Virus added pr 25 cm² flask

IPNV: 400 PFU/flask.

10 HSMIV: HSMI 1004.06 ALV702 1p 20/9-06. 150 ul/flask.

CMS: Isolate 1004.06 "CMS" 1p 20/9-06. 150 ul/flask.

Irido virus: diluted homogenate (1:1000) 90 ul/flask was added.

15

Virus	Cells	Temp	BrdU	Results				
				Day 4	Day 7	Day 10	Day 14	Day 21
-	GF-1	15 °C	no	-	-	-	-	-
-	GF-1	15 °C	yes	-	-	-	-	-
HSMIV	GF-1	15 °C	no	-	+	+	++	+++
HSMIV	GF-1	15 °C	yes	-	+	+	+	++
CMS	GF-1	15 °C	no	-	+	+	++	+++
CMS	GF-1	15 °C	yes	-	+	+	+	++
-	BF-2	15 + 25 °C	no	-	-	-	-	-
-	BF-2	15 + 25 °C	no	-	-	-	-	-
-	BF-2	15 + 25 °C	yes	-	-	-	-	-
IPNV	BF-2	15 °C	no	++	+++	+++	na	na
IPNV	BF-2	15 °C	yes	++	+++	+++	na	na
Irido(RSIV)	BF-2	25 °C	no	-	+	+++	na	na
Irido(RSIV)	BF-2	25 °C	yes	-	-	-	-	-

+ = weak infection +++ = fully developed infection

Conclusions / findings:

IPNV (RNA virus) infection was not inhibited by BrdU.

Irido (DNA virus) infection was inhibited by BrdU.

- 5 HSMI virus infection in early stages (up to 10 days) was not inhibited by BrdU. Beyond 14 days of incubation, more vacuoles were observed in flasks without BrdU. This is possibly due to inhibited cell division when BrdU is present, resulting in inhibited virus propagation.
- 10 CMS virus infection in early stages (up to 10 days) was not inhibited by BrdU. Beyond 14 days, more vacuoles were observed in flasks without BrdU. This is possibly due to inhibited cell division when BrdU is present, resulting in inhibited virus propagation.
- 15 The DNA synthesis inhibitor BrdU also affected the cells. At late timepoints BF-2 cells showed morphological changes due to the BrdU in the medium. GF-1 cells also responded to the BrdU in the medium. GF-1 cells did not divide, but grew large and stretched. Some cells had detached. This was observed in both uninfected and infected cell cultures.

20

Example 4: pH stability test of HSMIV and CMSV infected cell culture supernatants

Intention

- 25 The aim was to determine the pH stability of HSMI and CMS virus infected cell culture supernatants produced on GF-1 cells.

Materials

Virus controls

- IPNV: ALPHARMA AS, IPNV control (095), (-80 C), 30.05.-03.
- 30
 - PD virus: ALV-405, p6, 19.02.-07.
 - Relevant cell lines

Both virus controls were diluted to 5×10^5 TCID₅₀/ml in L-15.

Test materials

- 35
 - HSMI supernatant: HSMI 1004.06, ALV-702 1p, 20.09.06.
 - CMS supernatant: HSMI 1004.06, "CMS" 1p, 20.09.06.

- GF-1 cell cultures: 25cm² cell culture flasks with 4.5 x 10⁴ GF-1 cells (p43) per cm²
- Growth medium: L-15 (Sigma L-5520, lot 086K2414) with 10% FBS (Invitrogen 10101-145, lot 3105387 S, 1% L-glutamine (Sigma G-7513, lot 66K2435) and 0.1% Gentamicin (Sigma G-1397, lot 66K2433)

Methods

The pH in L-15 was adjusted to 5.0 and 3.0 by addition of 1.0M HCl, and the pH adjusted L-15 solutions were sterile filtrated (0.20µm). 350µl test substances or control virus were incubated in 5ml of the respective L-15 solutions in addition to normal L-15 (pH 7.2) for 4 hours at 15°C. After incubation, the pH was adjusted to 7.2 with 1.0M NaOH before 2.5ml of each solution were inoculated into two parallel 25cm² cell cultures in 5ml growth medium. All flasks were incubated at 15°C for three weeks and microscopically examined twice per week.

Results

Both HSMI and CMS virus were inactivated after incubation 4 hours with pH 3.0 at 15°C, whereas pH 5.0 had no effect compared with pH 7.2 on the development of CPE/vacuoles after inoculation of GF-1 cell cultures.

PD virus was not completely inactivated at pH 3.0 or 5.0, but the infection was significantly delayed in flasks inoculated with pH 5.0 treated virus, and even more delayed in the flasks inoculated with the pH 3.0 treated virus.

IPNV was not affected by treatment at pH 3.0 or 5.0.

All negative flasks remained negative throughout the observation period.

Microscopic observation.

pH	Flask	Result / day of conclusive recording				
		Neg. ctr.	PDV	IPNV	CMSV	HSMIV
7.2	A	- 21 d.pi.	+ 4 d.pi.	+ 4 d.pi.	+ 10 d.pi.	+ 10 d.pi.
	B	- 21 d.pi.	+ 4 d.pi.	+ 4 d.pi.	+ 10 d.pi.	+ 10 d.pi.
5.0	A	- 21 d.pi.	+ 7 d.pi.	+ 4 d.pi.	+ 10 d.pi.	+ 10 d.pi.
	B	- 21 d.pi.	+ 7 d.pi.	+ 4 d.pi.	+ 10 d.pi.	+ 10 d.pi.

ISAV(p3)	E	+	+	+	-	-	-	-	-	-
ISAV(p3)	F	+	+	+	-	-	-	-	-	-
Neg.homog.	G	-	-	-	-	-	-	-	-	-
Neg.homog.	H	-	-	-	-	-	-	-	-	-

+ = hemagglutination, - = no hemagglutination

Hemabsorption:

5

Infected cell cultures:

- HSMI p2 cultured in GF-1
- CMS p2 cultured in GF-1
- Negative control: Un-infected GF-1

10

Cytopathogen effect (CPE) was distinct in infected cell cultures.

Medium was removed and the cells were washed x 3 in PBS. Subsequently, 5 ml RBC (0.5%) was added to the flasks and incubated for 1 hour at 15°C.

15 The flasks were inspected under a microscope, and no hemabsorption was visible.

Example 6: Test of the CMS virus stock for contamination with other virus

The different cell lines were grown according to the literature and infected with 100µl CMS virus 1. passage stock. The cultures were observed for 28 days and appearance of cytopathogen effect was recorded. If a cytopathogen effect was observed, the culture was termed positive, if not the culture was termed negative.

Tests in cells sensitive to viruses pathogenic for salmonids			
Cell line Source	ATCC number	Viruses the cell line is permissive for	Result
BF-2 Bluegill fry, caudal trunk	CCL-91	Infectious Pancreatic Necrosis Virus Viral Haemorrhagic Syndrome Virus Infectious Haematopoietic Necrosis Virus (IHNV)	negative

BB Brown bullhead, Connective tissue and muscle	CCL-59	Infectious Pancreatic Necrosis Virus (IPNV) Channel Catfish Virus (CCV)	negative
RTG-2, Rainbow trout, gonadal tissue	CCL-55	Infectious Pancreatic Necrosis Virus (IPNV) Viral Haemorrhagic Syndrome Virus VHSV) Infectious Haematopoietic Necrosis Virus (IHNV) Spring Viremia of Carp Virus (SVCV)	negative
SHK-1, Salmon head kidney		Infectious Salmon Anemia Virus	negative
CHSE-214 Chinook salmon, embryo	CRL-1681	Infectious Pancreatic Necrosis Virus (IPNV) Pancreatic Disease Virus (PD)	negative
CCO Channel catfish, ovary	CRL-2772	Channel Catfish Virus (CCV)	negative
CHH-1 Chum salmon, heart fibroblast	CRL-1680		negative
FHM Fat head minnow Epithelial like	ECACC nr: 88102401	Infectious Pancreatic Necrosis Virus (IPN) Catfish Tumour Virus (CTV)	negative
GF-1 Grouper fin		Heart and Skeletal Muscle Infection Virus (HSMIV) Cardiomyopathy Syndrome	positive

		Virus (CMSV)	
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The results indicate that the CMS virus stock which is provided herein is free from contaminating virus of any of the species: Infectious Pancreatic Necrosis Virus (IPNV), Viral Haemorrhagic Syndrome Virus VHSV), Infectious Haematopoietic
5 Necrosis Virus (IHNV), Channel Catfish Virus (CCV), Spring Viremia of Carp Virus (SVCV), Infectious Salmon Anemia Virus, Pancreatic Disease Virus (PD), Catfish Tumour Virus (CTV), The results further indicate that the virus has phenotypic characteristics which are different from the characteristics of any of these viruses.

10 Example 7: PCR-analyses of the CMS virus stock using species specific primers

Virus isolates:
4,5x10E04 GF-1 cells/cm² was transferred to 2 x 175cm² cell flasks and 1 x 75cm²
flask. Both 175cm² flasks were inoculated with 500µl homogenate from fish with a
15 CMS diagnosis.

The CMSV positive flasks were freeze/thawed in 2 cycles 13 days post infection. At this point, the positive flasks showed severe CPE, while the negative control did not deviate from the normal morphology. The cell lysate was pulled through a 23g
20 and then a 25g syringe, before being sterile filtered (0,20µm). The lysate was then dispensed in 4,5 ml tubes and frozen at -80°C.

Positive controls:
Infectious Pancreatic Necrosis Virus (IPNV) and Grouper Nervous Necrosis Virus (GNNV) from cell culture, Salmonid Alphavirus 3 E2 gene, plasmid mini prep. The
25 GNNV was used as a positive control for nodaviruses. The primers used in the PCR reaction will also recognise other nodaviruses like Viral Nervous Necrosis virus (VNNV) from cod.

RNA isolation: RNA was isolated using TriZol procedure in accordance with protocol (Invitrogen).

30

RT-reaction:
The following reactions were set up:
- 6µl RNA template (1,7µg CMSV RNA 202,74ng IPNV RNA or 753,18ng GNNV RNA)
35 - 1µl Random Hexamers (50ng/µl)

- 1µl Annealing Buffer

The reaction were incubated at 65°C for 5min, and then on ice for at least a minute.

5

To the reactions was then added:

- 10µl 2x First-Strand Reaction Mix
- 2µl SuperScript™ III/RNaseOUT Enzyme Mix

10 The reactions were then incubated at:

- 25°C – 10min
- 50°C – 50min
- 85°C – 5min

15 **PCR reaction:**

- 15µl GoTaq™ Master Mix
- 2x1µl primer fwd/rev (25µM per primer)
- 5µl cDNA templat (from a 1:10 dilution)
- 2µl dH₂O
- 1µl GoTaq pol. enzyme

20

The reactions went through the following temperature cycle:

- 25
- 95°C – 5min
 - 95°C – 30sek
 - 50°C – 30sek
 - 72°C – 1min
 - 72°C – 10
- } 30 cycles

PCR setup and results:

30

PCR Tube	Temp late	Primer sequence	Target size (bp)	PCR test result
1	CMS	CGTCACTTTCACCAGCGACTCCCAGACG (SEQ ID NO: 1)	305	negative
		GGATCCATTCGGATGTGGCGTTGCTATG G (SEQ ID NO: 2)		
2	CMS	CTGCGGTGTAGACATCAAAG (SEQ ID NO: 3)	222	negative
		TGCAGTTCCTCGTCCATCC (SEQ ID NO: 4)		

		4)		
3	CMS	GGATTTGGACGTGGGACCAA (SEQ ID NO: 5)	891	negative
		CGGATGACCCGGTTAGTTTTC (SEQ ID NO: 6)		
4	none	CGTCACTTTCACCAGCGACTCCCAGACG (SEQ ID NO: 1)	305	negative
		GGATCCATTCGGATGTGGCGTTGCTATG G (SEQ ID NO: 2)		
5	none	CTGCGGTGTAGACATCAAAG (SEQ ID NO: 3)	222	negative
		TGCAGTTCCTCGTCCATCC (SEQ ID NO: 4)		
6	SAV3	CGTCACTTTCACCAGCGACTCCCAGACG (SEQ ID NO: 1)	305	positive
		GGATCCATTCGGATGTGGCGTTGCTATG G (SEQ ID NO: 2)		
7	IPNV	CTGCGGTGTAGACATCAAAG (SEQ ID NO: 3)	222	positive
		TGCAGTTCCTCGTCCATCC (SEQ ID NO: 4)		
8	GNNV	GGATTTGGACGTGGGACCAA (SEQ ID NO: 5)	891	positive
		CGGATGACCCGGTTAGTTTTC (SEQ ID NO: 6)		

Conclusion: All CMS samples and the no template controls were negative in the PCR-tests, while the positive controls were positive. The SAV specific primers 5 (SEQ ID Nos: 1 and 2) recognise all known types of salmonid alphavirus (SAV), the GNNV specific primers (SEQ ID Nos: 5 and 6) recognise most VNN, and the IPNV specific primers recognise most of the IPNV serotypes. These tests indicate that the CMS virus isolates are not contaminated by IPNV, nodavirus or salmonid alphavirus. The results further suggest that the CMS virus does not have close

resemblance to any of these viruses and that it has genotypic characteristics which are different from the genotypic characteristics of any of these viruses.

Example 8: Electron micrograph

- 5 Cells were grown in slide flasks and infected with CMS virus according to the invention. The cells were washed with PBS and fixed with 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer. They were further post-fixed with 2% osmium tetroxide and 1.5% potassium ferrocyanide in 0.1M sodium cacodylate buffer, followed by staining with 1.5% uranyl acetate in distilled water. Upon dehydration
10 though increasing alcohol concentrations the specimens were embedded in Epon™ plastic resin. Ultrathin sections were cut and post-stained with 0.2% lead citrate. Sections were examined in a Philips™ CM 100 at 80 kv.

Results: Examination of the electron micrographs revealed the presence of spherical virus-like particles structures having a diameter of approximately 70 nm.

15

Example 9: CMSV cultivation in cell factory (CF-1) and subsequent isolation of virus

In order to achieve a high virus titer, putative CMSV was cultivated in GF-1 cells in a cell factory (CF-10 + CF-1). A CMS positive tissue homogenate was used as inoculum.

Since isolation of CMSV after the 2nd passage has been difficult to identify (no CPE), a homogenate was chosen as inoculum in the cell factory (p1). Further cultivation after the isolation (p2) procedure would then hopefully yield the expected CPE in GF-1 cells (vacuolization).

20 Experimental and Results

Homogenate: The homogenate was made of 4 hearts, Atlantic salmon – *Salmo salar* – diagnosed with CMS, each weighing approx. 10g. The salmons were all localized to Vindsvik, Rogaland, Norway.

- Bulbus arteriosus was removed and the remaining atrium and ventricle was cut in
25 to smaller pieces with a sterile scalpel. The tissue was then crushed, in combination with quartz sand and 80ml L-15 media, using a pestle. This step was followed by centrifugation at 2000xg for 10min, and filtration of the supernatant (0,2 µm).

Growth in cell factory:

0dpi:

GF-1 cells were trypsinated and transferred to one CF-1 and one CF-10 in a concentration of $4,5E4$ cells/cm². 9 ml of CMSV homogenate was added to the cell suspension upon transfer.

7dpi:

Examination revealed developing vacuoles and slightly toxic effect.

15dpi:

No changes.

10 37dpi:

At this point, CPE could be observed in almost all cells, and cell culture supernatant was harvested from both CF-1 and CF-10 (approx. 2L).

Rate-zonal density gradient:

15 The supernatant was centrifuged at 17700xg in a JA-10 rotor for 30 min. The pellet was re-suspended in 4 ml L-15 media and frozen at -80°C.

Refractive index of the supernatant was measured and found to be 1,3360, the equivalent to a density of 1,0060g/cm³. This step was performed because the media in a GF-1 cell culture tend to be fairly viscous.

20 The supernatant was spun at 100000xg for 3h in a JS 24.38 rotor, which was capable of spinning a volume of 6x38ml at a time. For every round in the centrifuge the 6 pellets were re-suspended in a total of 150µl PBS and frozen at -80°C.

25 Sucrose density gradients:

- 6ml 36% sucrose in PBS w/v
- 6ml 42% sucrose in PBS w/v
- 6ml 48% sucrose in PBS w/v
- 6ml 54% sucrose in PBS w/v
- 30 - 6ml 60% sucrose in PBS w/v

After layering the different sucrose concentrations in the tube, it was incubated at 4°C over night.

600µl of pellet material (after ultra-centrifugation) was diluted to 5ml in PBS and loaded on to the gradient, which subsequently was spun at 100000xg at 4°C for 35 16h. When the gradient was examined after run, a band was clearly visible in the

denser part of the gradient. Also, two weaker bands were visible in the upper, less dense part of the gradient.

Fractions were collected in 2 ml aliquots from the bottom of the tube, and the refractive index was measured in each fraction (Table 1).

5

Table 1:

Fraction	Refractive index	Density (g/cm³)	CPE
1	1,4132	1,2186	No
2	1,4113	1,2079	No
3	1,4073	1,1972	No
4	1,4015	1,1868	No
5	1,3964	1,1663	Yes
6	1,3925	1,1562	No
7	1,3899	1,1464	No
8	1,3876	1,1366	No
9	1,3859	1,1366	No
10	1,3841	1,1366	No
11	1,3822	1,1270	No
12	1,3806	1,1270	No
13	1,3788	1,1175	No
14	1,3724	1,1082	Yes
15	1,3588	1,0678	No
16	1,3474	1,0361	No
17	1,3434	1,0259	No

Each fraction was diluted to 35ml PBS and then spun at 100000xg at 4°C for 3h.

10 Pelleted material was re-suspended in 150µl PBS.

75µl of each fraction was used as inoculums in respective 25cm² cell flasks with 4.5x10E04 GF-1 cells/cm². Cells were incubated at 15°C and inspected routinely.

The only flasks showing cells with CPE were the ones inoculated with fraction 5 and 14. The CPE was characterized by vacuolated cells, and was observed from 7

15 dpi with increasing intensity.

Conclusions/findings:

After removing most of the cellular debris from a cell culture supernatant (CF-10, with GF-1 inoculated with CMS positive material) by conventional centrifugation, high-speed centrifugation was applied in order to concentrate the viral load in the

supernatant. After concentrating the material, a rate-zonal density gradient centrifugation was performed. The resulting gradient showed two bands which, in a following cell culture experiment, gave a CPE with the usual characteristics (both time and morphology) associated with CMS positive material in GF-1 cells.

5

Example 10: Preparation of antigen for vaccines and immunization of rabbits

CMS-material stock was prepared from homogenates of heart tissue from fish diagnosed with CMS. The heart tissue (1,3 g) was homogenized in a mortar with the addition of sand and was suspended in (3,2 ml) L-15 (Sigma L-5520, lot 64K22413) with 10% FBS (Sigma F-3885, lot 012K84132), 1% L-glutamine (Sigma G-7513, lot 24K2431) and 0.1% Gentamicin (Sigma G-1397, lot 113K2312). The resulting suspension was centrifuged for 10 minutes at 3000xg. The supernatant was filtered through a 45 µm syringe filter (sartorius 17829) and was subsequently kept frozen at -80°C.

Antigen was prepared with the use of GF-1 cells (ATCC PTA-859) infected with 1st passage stock of CMS-material stock. The cells were seeded at 4.5×10^4 cells/cm² and were infected with 400 µl 1st passage of CMS-material stock per 75 cm². The cell cultures were incubated for 14-18 days at 15°C until a cytopathogen effect (CPE) was visible throughout the entire culture. The supernatant was removed and collected in 50 ml vials and subsequently spun at 3000xg for 30 minutes. The supernatant was transferred into transparent centrifuge tubes and spun at 100000xg for two hours. The resulting pellet (A) was re-suspended in phosphate buffered saline (PBS).

The cells were harvested by scraping with a rubber policeman and were transferred to sample vials. The cells were then homogenised by passing the material 5 times through a 21G syringe 5 times through a 23G syringe and, finally, 4-5 times through a 25G. The homogenate was centrifuged for 30 min. at 3000xg. The supernatant was transferred to centrifuge tubes (clear transparent) and was centrifuged for 2 hours at 100000 xg. The resulting pellet (B) was re-suspended in PBS. The re-suspended pellets (A) and (B) were combined.

Inactivation was performed by adding formalin in relative amounts of 2000 ppm (2 g/liter). The antigen was transferred to new tubes and incubated at room temperature for 48 hours. Finally, the antigen was dialysed against PBS in a cassette, cut off value being 10.000. PBS was changed three times, the antigen being left overnight in the last change of PBS.

Protein	Dilution	mg/ml	OD-
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concentration was determined using the DC protein assay from BioRad: Sample			Blank
CMS	4	0,767002	0,0305
	16	1,056199	0,0105

CMS = 0.8 mg/ml

- 5 A water-in-oil fish vaccine was prepared containing a 1:5 dilution of the antigen prepared as described above per ml.

Clinical trial:

A clinical trial was run using ca 30 g Atlantic salmon post smolts. The trial was run in salt water at 12 °C. Each fish was injected with a 0.1 ml dose of vaccine. Six weeks later the fish were challenge intramuscularly with 0.2 ml of 1.st passage. Samples were collected at 6, 8, 10 and 12 weeks post challenge, and changes in the atrium and ventricle of the heart was determined by histological analyses using standard methods.

15

The results show that the fish vaccinated with the CMS vaccine is protected against histopathological changes resembling CMS. Some fish become positive for histopathological changes when challenged with CMSV, but the changes disappear 10-12 weeks after challenge. This shows that a vaccine containing inactivated CMS virus can protect against the development of CMS. There was no significant reduction of histopathological changes in fish which had received a HSMI vaccine as compared to PBS controls.

The cardiomyopathy syndrome virus (CMS) has been deposited with the European collection of cell cultures on March 29, 2007, and was granted accession number 07032902.

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Claims

1. A fish virus, which when injected intraperitoneally or intramuscularly in doses of 0.1 – 0.2 ml/fish of an infected GF-1 cell homogenate prepared from 1st passage of the virus, has the ability to induce the symptoms and disease of Cardiomyopathy Syndrome (CMS) and wherein:

- i) said virus is the strain deposited under the Budapest Treaty with the European Collection of Cell Culture (ECACC), Health Protection Agency, Porton Down, Salisbury, Wiltshire (UK), SP4 0JG UK on the 29 March 2007 under accession number 07032902 or a strain with the same genotypic and/or phenotypic characteristics, or
- ii) said virus reacts with immune serum from rabbit raised against the strain deposited under ECACC accession number 07032902.

2. The virus according to claim 1, wherein said virus is obtained from the strain deposited under the Budapest Treaty with the European Collection of Cell Culture (ECACC), Health Protection Agency, Porton Down, Salisbury, Wiltshire (UK), SP4 0JG UK on the 29 March 2007 under accession number 07032902.

3. The fish virus of claim 2, wherein said virus is obtained from said deposited strain by passage on a cell line.

4. The fish virus according to any one of claims 1-3, wherein said virus when provided in a tissue homogenate from an infected fish, is incapable of growing in any cell line from the group consisting of: BF-2 (Bluegill fry, caudal trunk), BB (Brown bullhead, connective tissue and muscle), RTG-2 (Rainbow trout, gonadal tissue), SHK-1 (salmon head, kidney), CHSE-214 (Chinook salmon, embryo), CCO (Channel catfish, ovary), CHH-1 (Chum salmon, heart fibroblast) and FHM (Fat head minnow, epithelial like).

5. The fish virus according to one of claims 1-4, said virus being inactivated by incubation with 10% v/v chloroform for 30 minutes at 20°C with continuous shaking.

6. The fish virus according to any one of claims 1-4, said virus being inactivated by incubation at 70°C for 30 minutes while being insensitive to incubation at 60°C for 30 minutes.

7. The fish virus according to any one of claims 1-6, said virus being insensitive to incubation with 100 µg/ml DNA synthesis inhibitor BrdU.
8. The fish virus according to any one of claims 1-7, said virus being in an isolated form or substantially or essentially free of other viral or microbial material.
9. The fish virus according to any one of claims 1-4, said virus being attenuated or inactivated.
10. A cell line comprising the fish virus as defined in any one of claims 1-9.
11. The cell line according to claim 10, wherein the cell line is derived from *Epinephelus coioides*.
12. The cell line according to claim 10, wherein said cell line is GF-1 (ATCC PTA-859).
13. A vaccine comprising a virus as defined in claim 9, or an antigenic component or antigenic part of said virus.
14. The vaccine according to claim 13, said vaccine comprising an amount of antigen, which is in the range of 0.05-1.5 mg/ml, or said vaccine being for administration in dosages comprising an amount of antigen corresponding to a TCID₅₀ of 10⁵-10¹⁰ per dosage.
15. The vaccine according to claim 13 or 14, in a formulation comprising an adjuvant.
16. The vaccine according to claim 14 or 15 further comprising a suitable pharmaceutical carrier.
17. The vaccine according to any one of claims 13 to 15 in a formulation comprising an antigen from a bacterial source, an antigenic material obtained from a viral source other than the fish virus as defined in claims 1-9, an antigenic material obtained from a parasitical source, or an antigenic material obtained from a fungal source.
18. The vaccine according to claim 17, wherein said antigen from a bacterial source is selected from the group consisting of live, attenuated or killed bacteria of the species *Piscirickettsias sp.*, *Aeromonas sp.*, *Vibrio sp.*, *Listonella sp.*, *Moritella viscosa*, *Photobacterium damsela*, *Flavobacterium sp.*, *Yersinia sp.*, *Renibacterium sp.*, *Streptococcus sp.*, *Lactococcus sp.*, *Leuconostoc sp.*, *Bifidobacterium sp.*, *Pediococcus sp.*, *Brevibacterium sp.*,

Edwarsiella sp., *Francisella sp.*, *Pseudomonas sp.*, *Cytophaga sp.*, *Nocardia sp.*, *Mycobacterium sp.*, parts or subunits of these bacteria, and any combination hereof.

19. The vaccine according to claim 17, wherein said antigenic material obtained from a viral source other than the fish virus as defined in any of claims 1-9 is from a virus selected from the group consisting of Viral Hemorrhagic Septicemia Virus (VHSV), Infectious Hematopoietic Necrosis virus (IHNV), Infectious Pancreatic Necrosis Virus (IPNV), Spring Viremia of Carp (SVC), Channel Catfish Virus (CCV), Infectious Salmon Anaemia virus (ISAV), pancreatic disease virus (SPDV), Iridovirus, and heart and skeletal muscle inflammation virus (HSMIV), parts or subunits of any one of these viruses, and combinations hereof.

20. The vaccine according to claim 17 or 19, wherein said antigenic material obtained from a viral source other than the fish virus as defined in any of claims 1-9 is selected from the group consisting of Glycoprotein of Viral Hemorrhagic Septicemia Virus (VHSV), nucleoprotein of Viral Hemorrhagic Septicemia Virus (VHSV), glycoprotein of Infectious Hematopoietic Necrosis virus (IHNV), nucleoprotein structural proteins of Infectious Pancreatic Necrosis Virus (IPNV), G protein of Spring Viremia of Carp (SVC), and a membrane-associated protein, tegumin or capsid protein or glycoprotein, of Channel Catfish Virus (CCV) and antigenic fragments of any of one of these proteins.

21. The vaccine according to claim 17, wherein said parasitic source is selected from the group consisting of *Lepeophtheirus Sp.*, *Caligus Sp.*, and *Ichthyophthirius Sp.*, parts of any of these parasites, and combinations hereof.

22. The vaccine according to claim 17, wherein said fungal source is selected from the group consisting of *Saprolegnia Sp.*, *Branchiomyces sanguinis*, *Branchiomyces demigrans* and *Ichthyophonus hoferi*.

23. The vaccine according to any one of claims 13 to 22, said vaccine being formulated for administration to a fin fish.

24. The vaccine according to any one of claims 13 to 22, said vaccine being formulated for administration to a telostei.

25. The vaccine according to any one of claims 13 to 24, said vaccine being formulated for administration by a route selected from the group consisting of: Bath, immersion, intraperitoneal injection, intramuscular injection and oral administration.

26. A feed comprising a vaccine according to any one of claims 13 to 25.

27. An antiserum or isolated antibody, which selectively binds to a virus as claimed in any one of claims 1-9.

28. The isolated antibody according to claim 27 comprising a marker.

29. A method of isolating or producing a virus as defined in any of claims 1-9, said method comprising

- i) producing a homogenate of a tissue from a fish suffering from/showing the symptoms of Cardiomyopathy Syndrome (CMS);
- ii) inoculating a cell culture of a suitable cell line using the homogenate of step i); and
- iii) isolating virus particles from said cell or from the medium in which the cell is cultured.

30. A virus as defined in claim 9 or an antigenic part of said virus for use in fish medicine.

31. A virus or an antigenic part of a virus as claimed in claim 30 for use in the treatment of Cardiomyopathy Syndrome (CMS) in fish.

32. A virus as defined in claim 9 or an antigenic part of said virus for use in prevention of Cardiomyopathy Syndrome (CMS) in fish or for reducing the viral load in a fish or for reducing the incidence of CMS in a population of fish or for treatment of Cardiomyopathy Syndrome (CMS) in fish or for reducing the incidence or severity of lesions caused by Cardiomyopathy Syndrome Virus in fish.

33. Use of the virus as defined in claim 9 or an antigenic part of said virus for the manufacture of a medicament for prevention of Cardiomyopathy Syndrome (CMS) in fish or for reducing the viral load in a fish or for reducing the incidence of Cardiomyopathy Syndrome in a population of fish or for treatment of Cardiomyopathy Syndrome in fish or for reducing the

incidence or severity of lesions caused by Cardiomyopathy Syndrome Virus in fish.

34. A vaccine as defined in any one of claims 13 to 25 for prevention of Cardiomyopathy Syndrome in fish, for reducing the viral load in a fish, for reducing the incidence of Cardiomyopathy Syndrome in a population of fish or for reducing the incidence or severity of lesions caused by Cardiomyopathy Syndrome Virus in fish.

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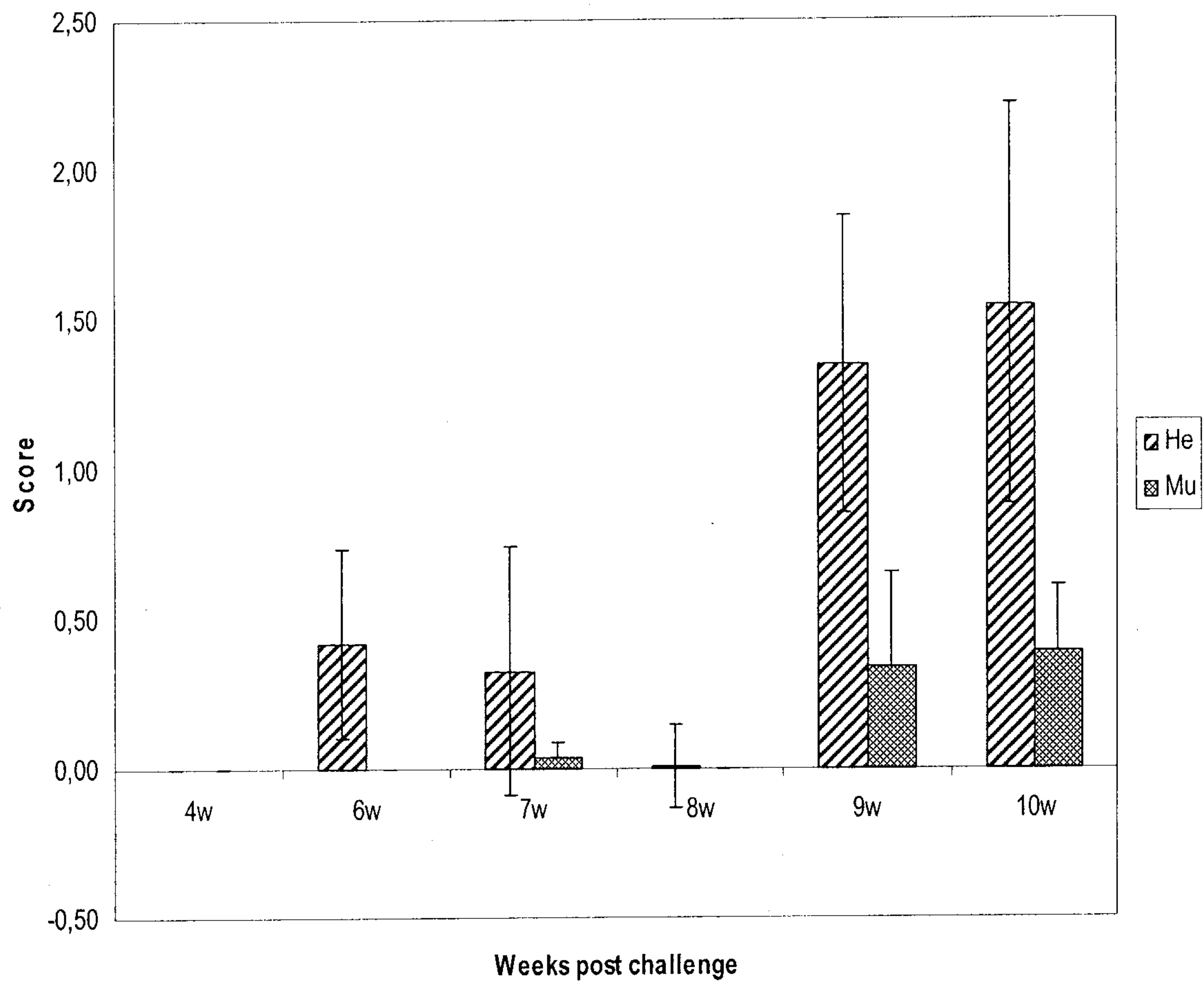


Fig. 1

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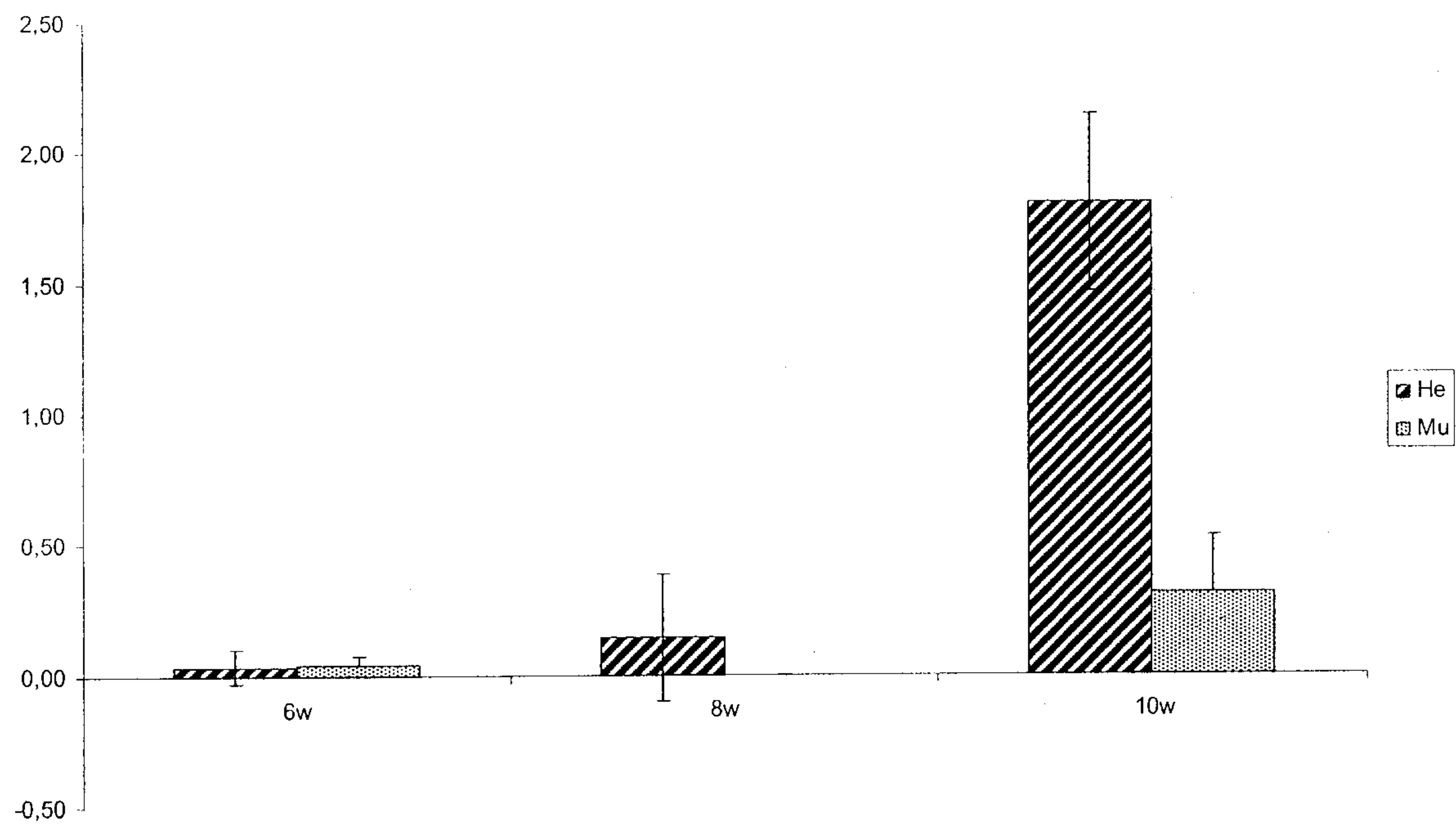


Fig. 2

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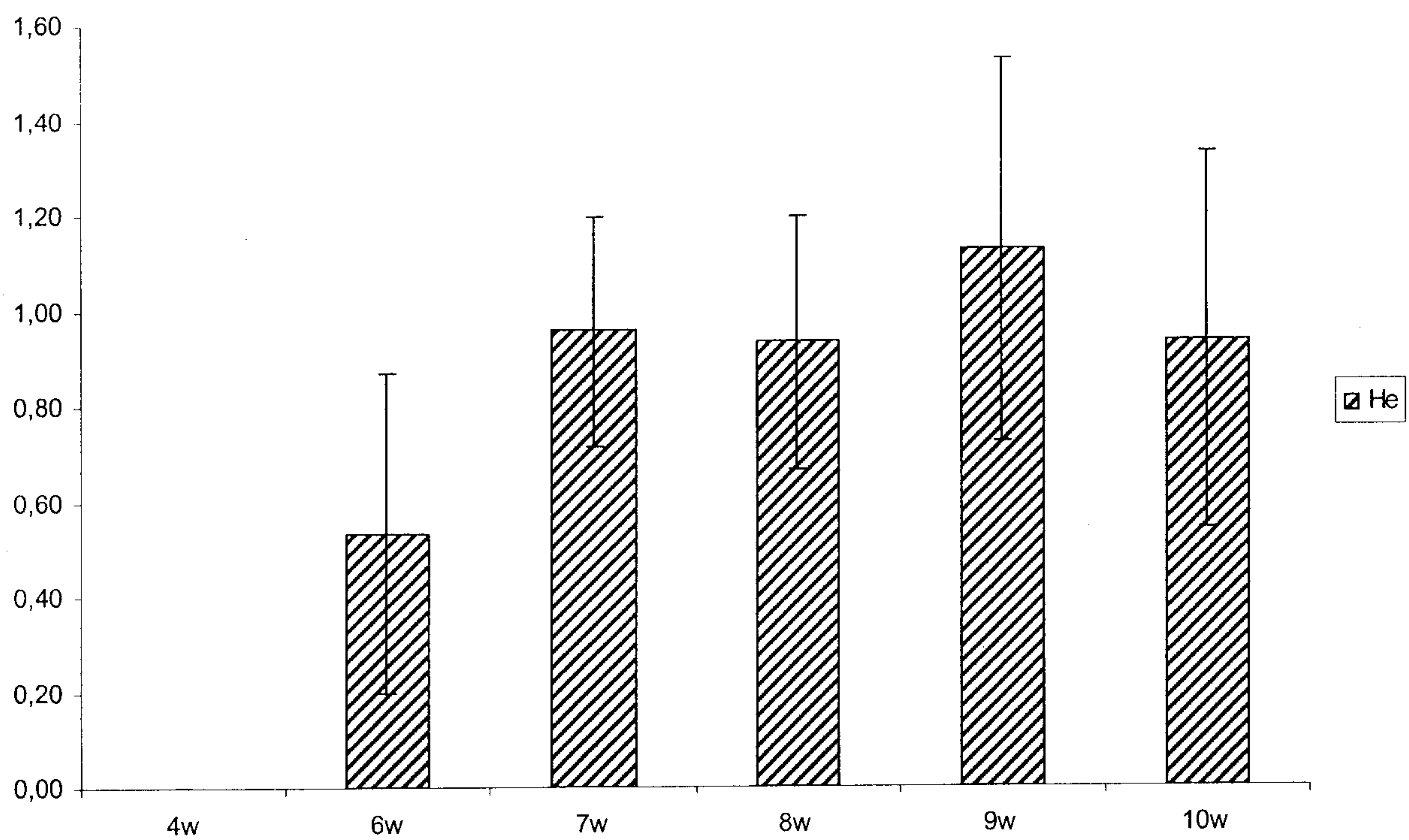


Fig. 3

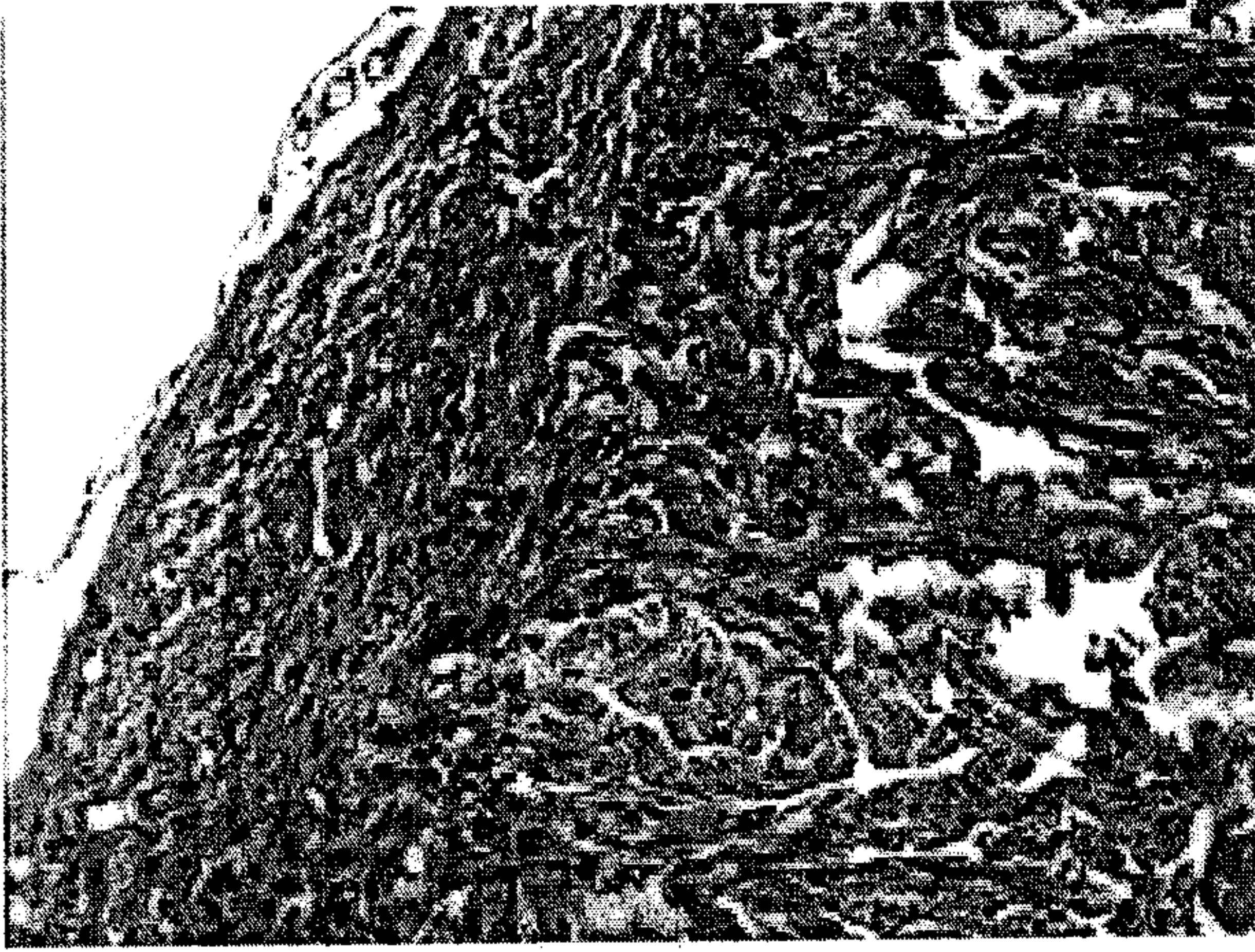
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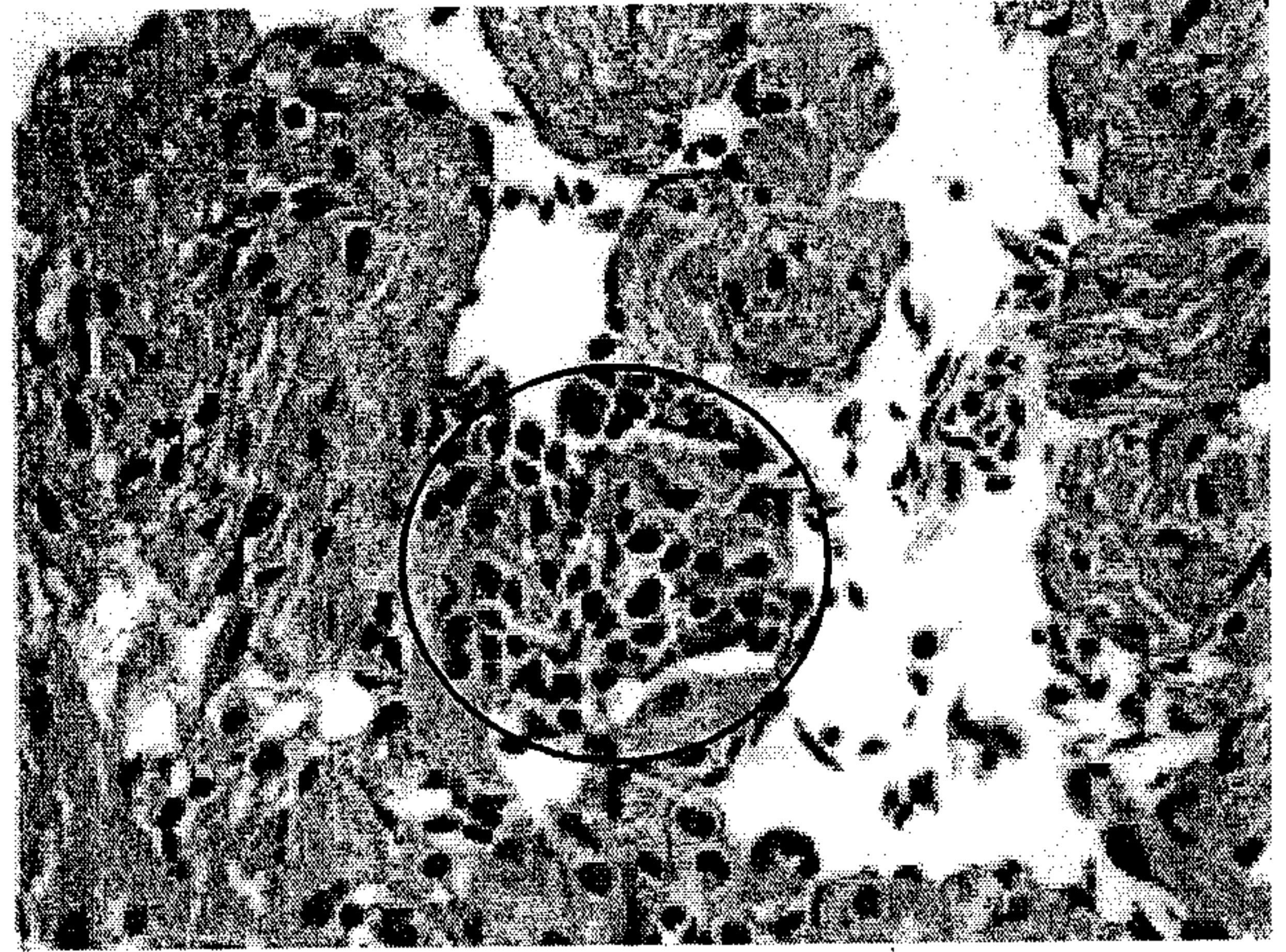
Fig. 4

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A.



B.



C.



D.

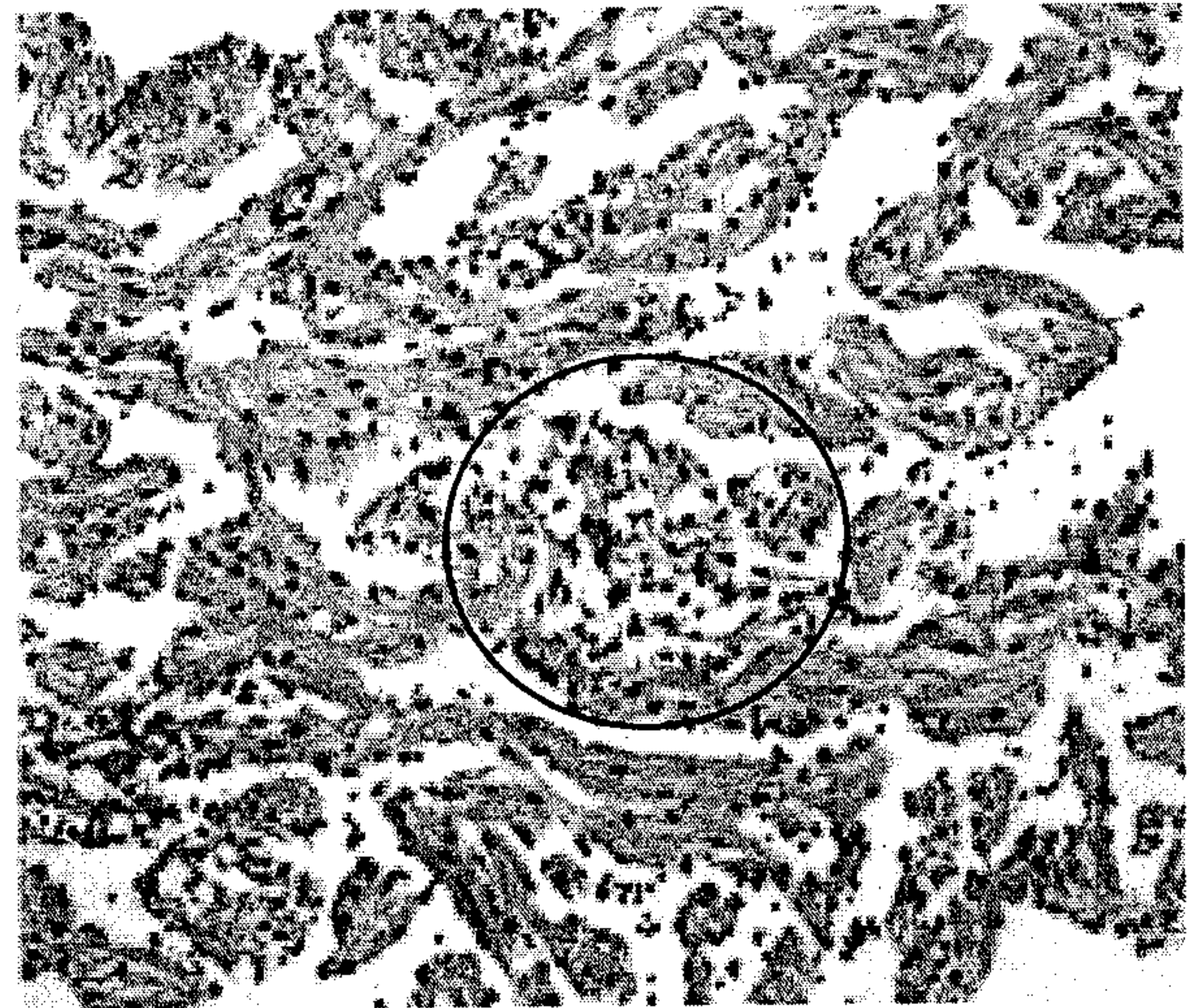
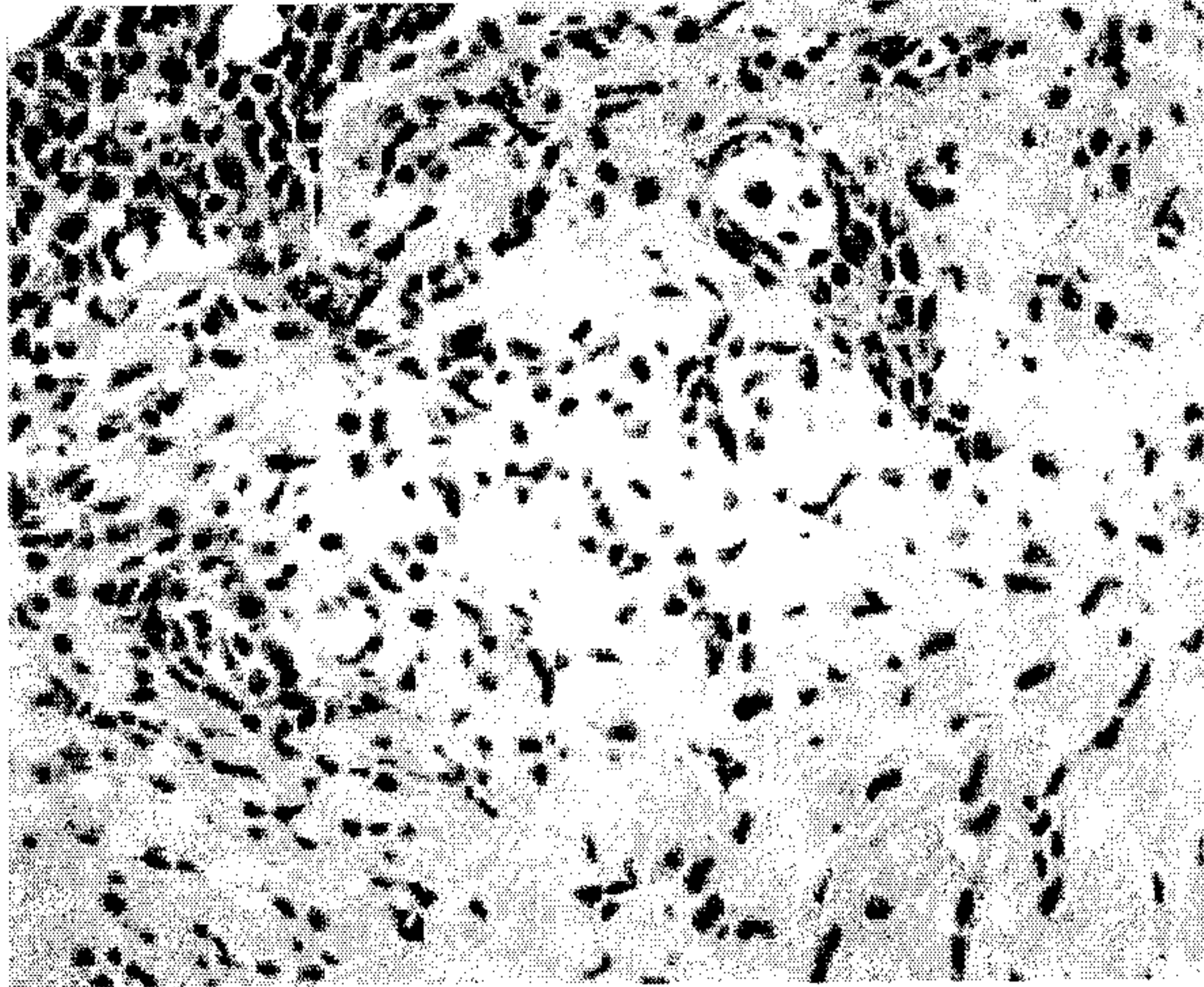


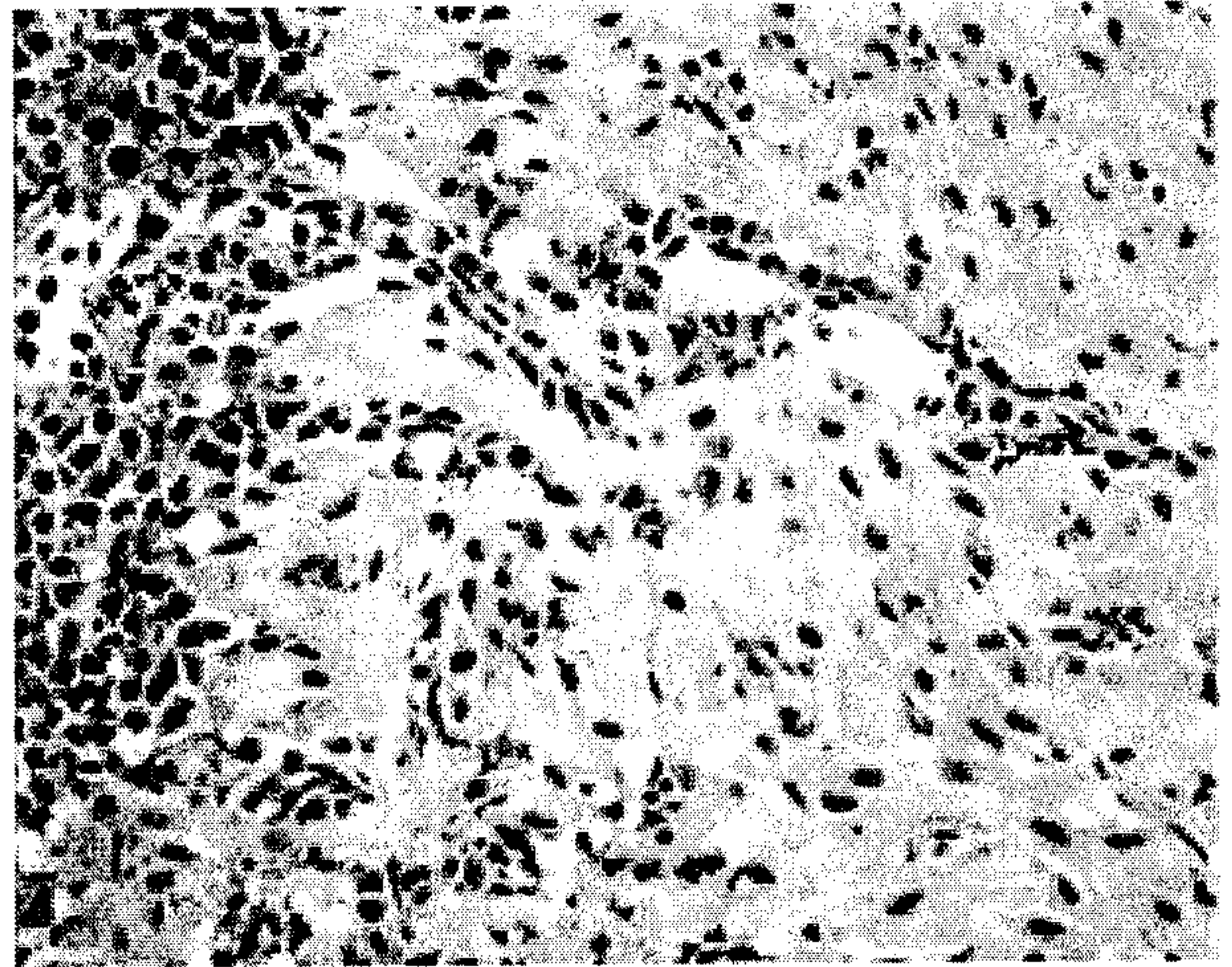
Fig. 5

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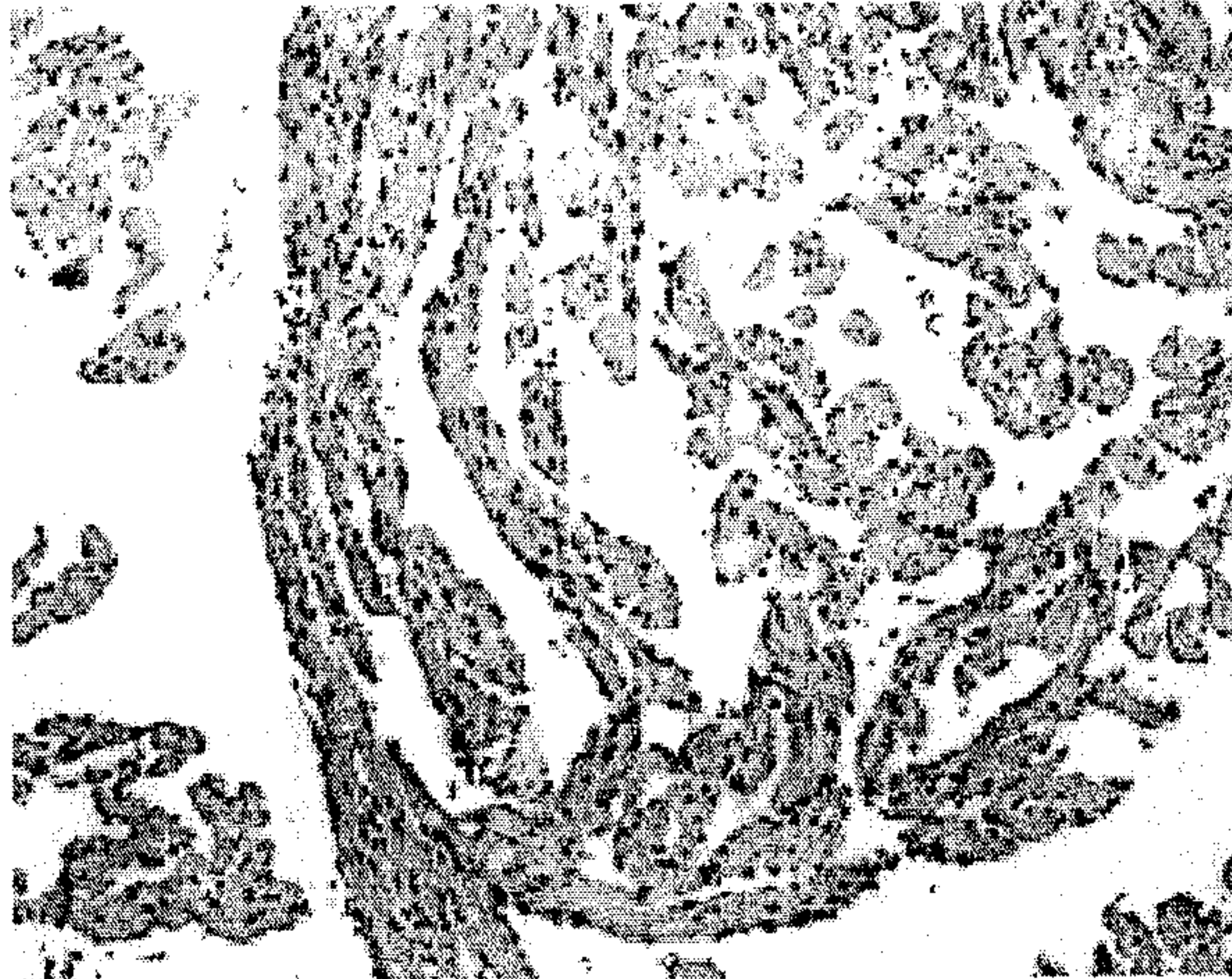
A.



B.



C.



D.

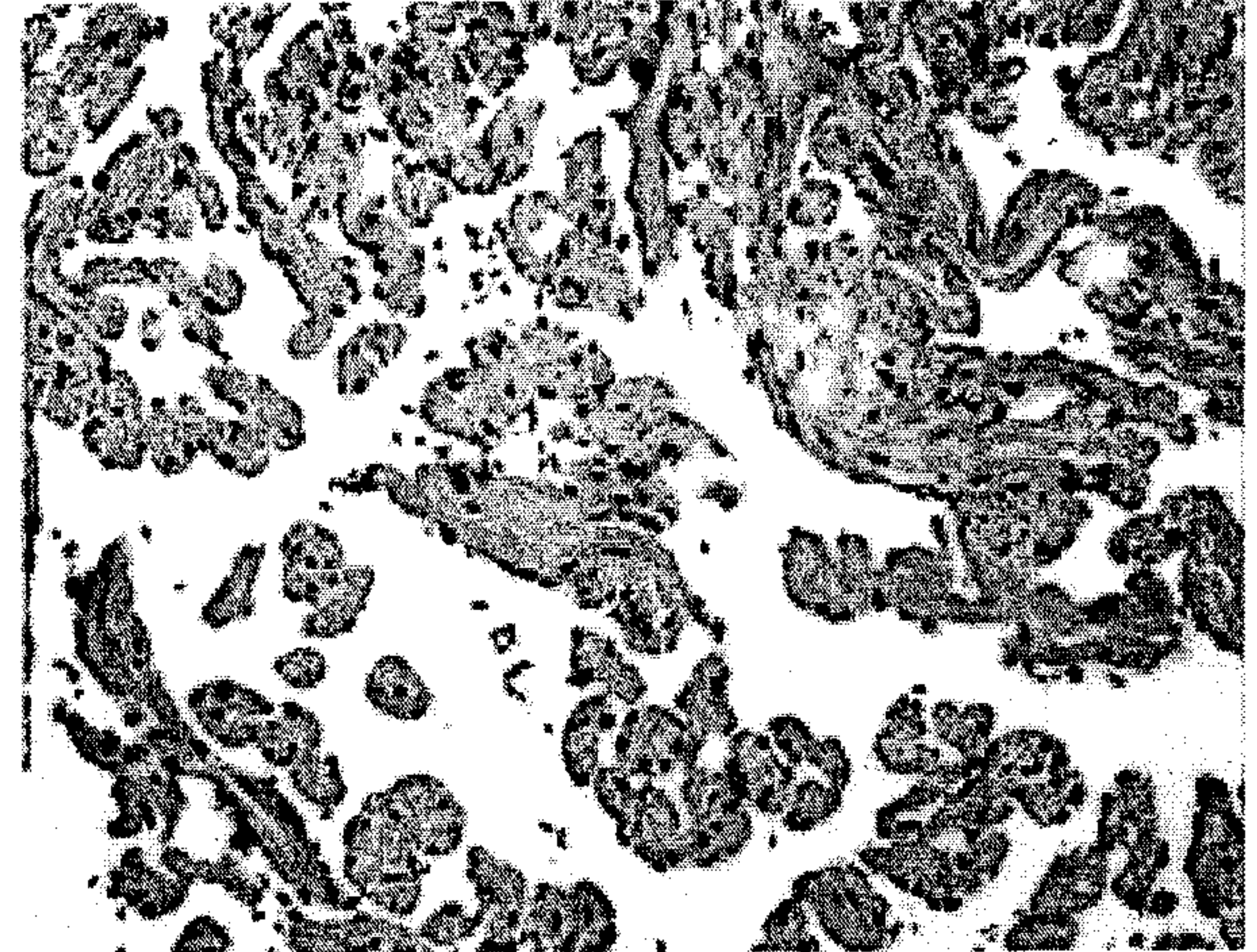


Fig. 6

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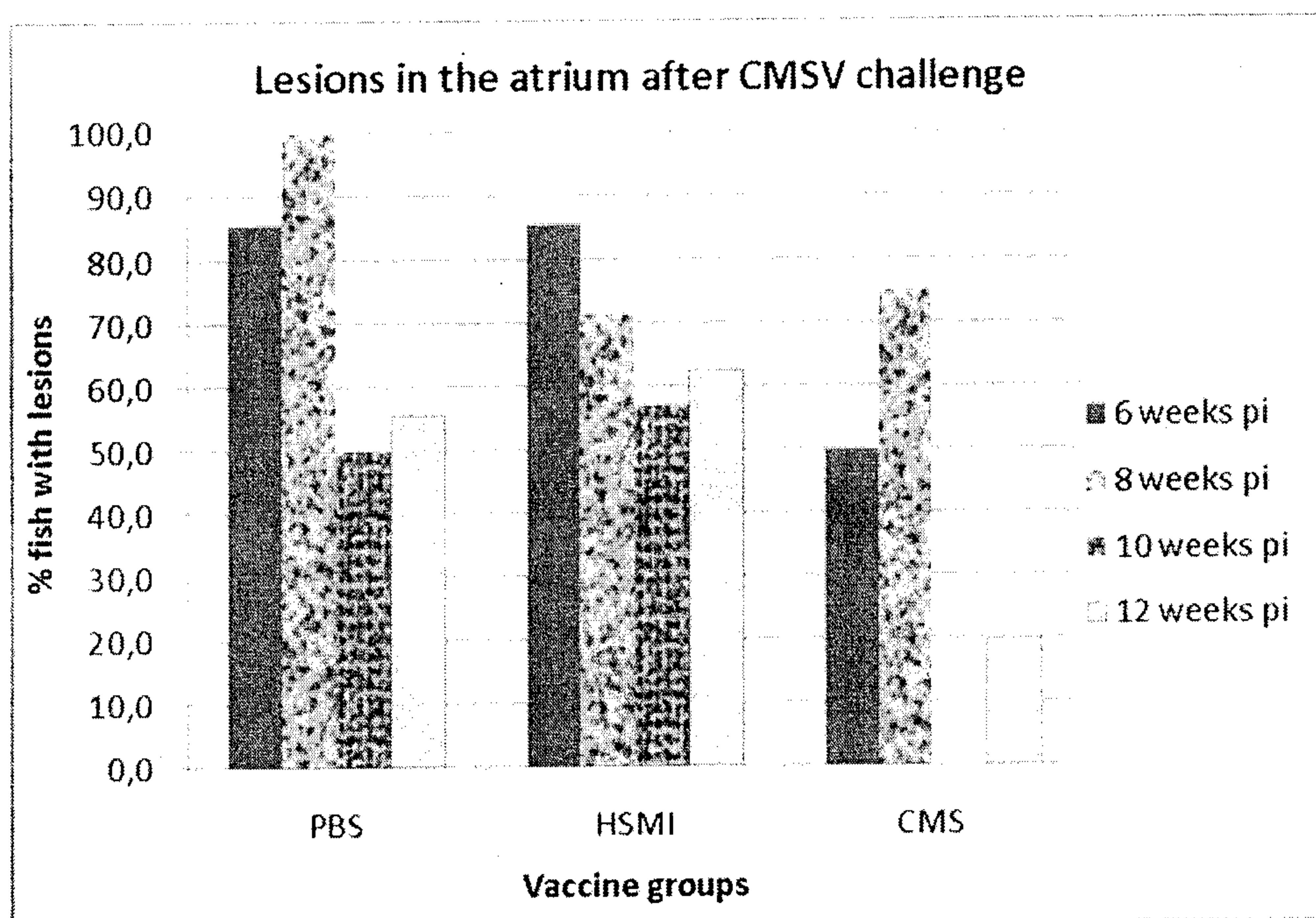


Fig. 7

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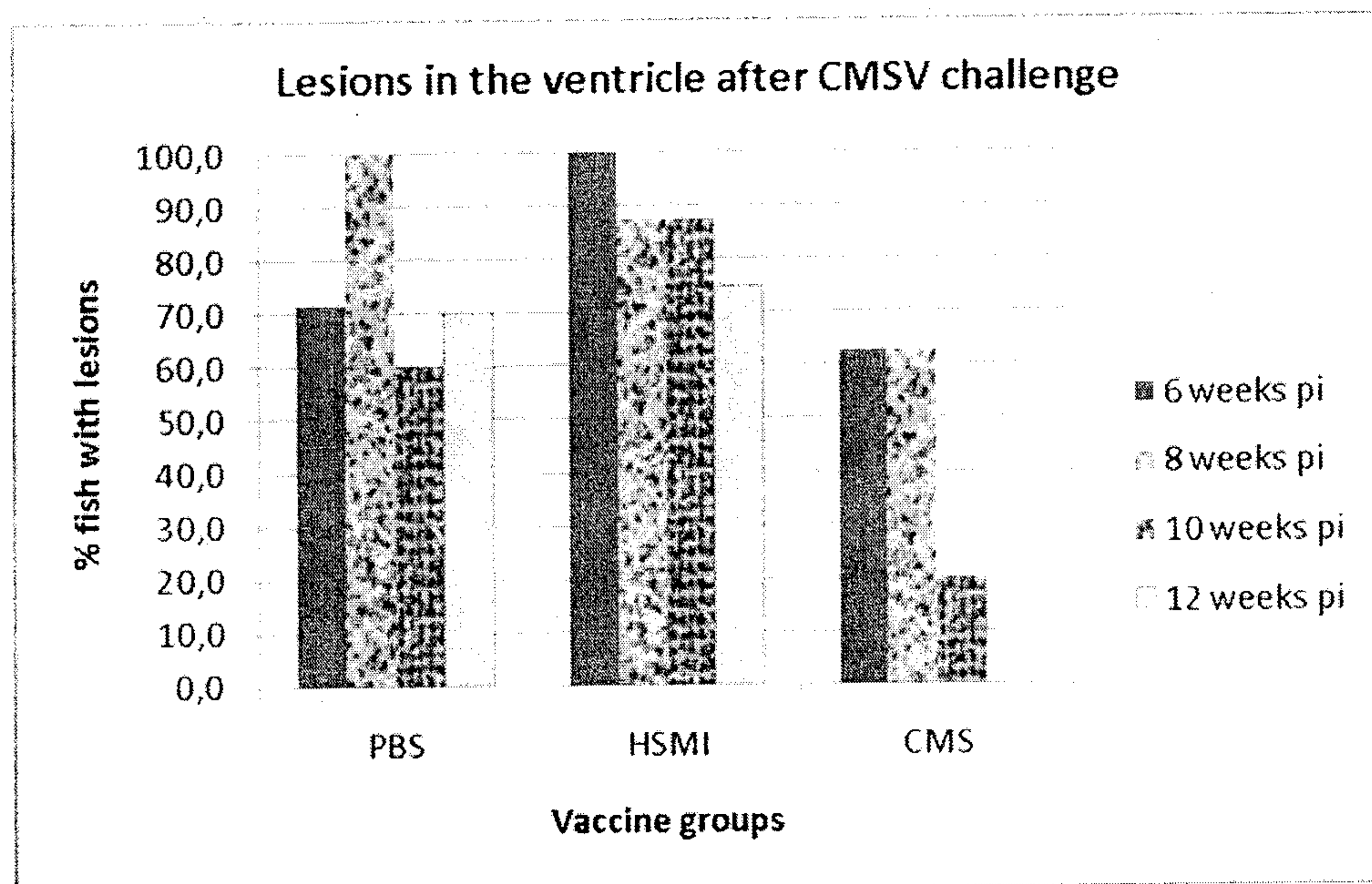


Fig. 8

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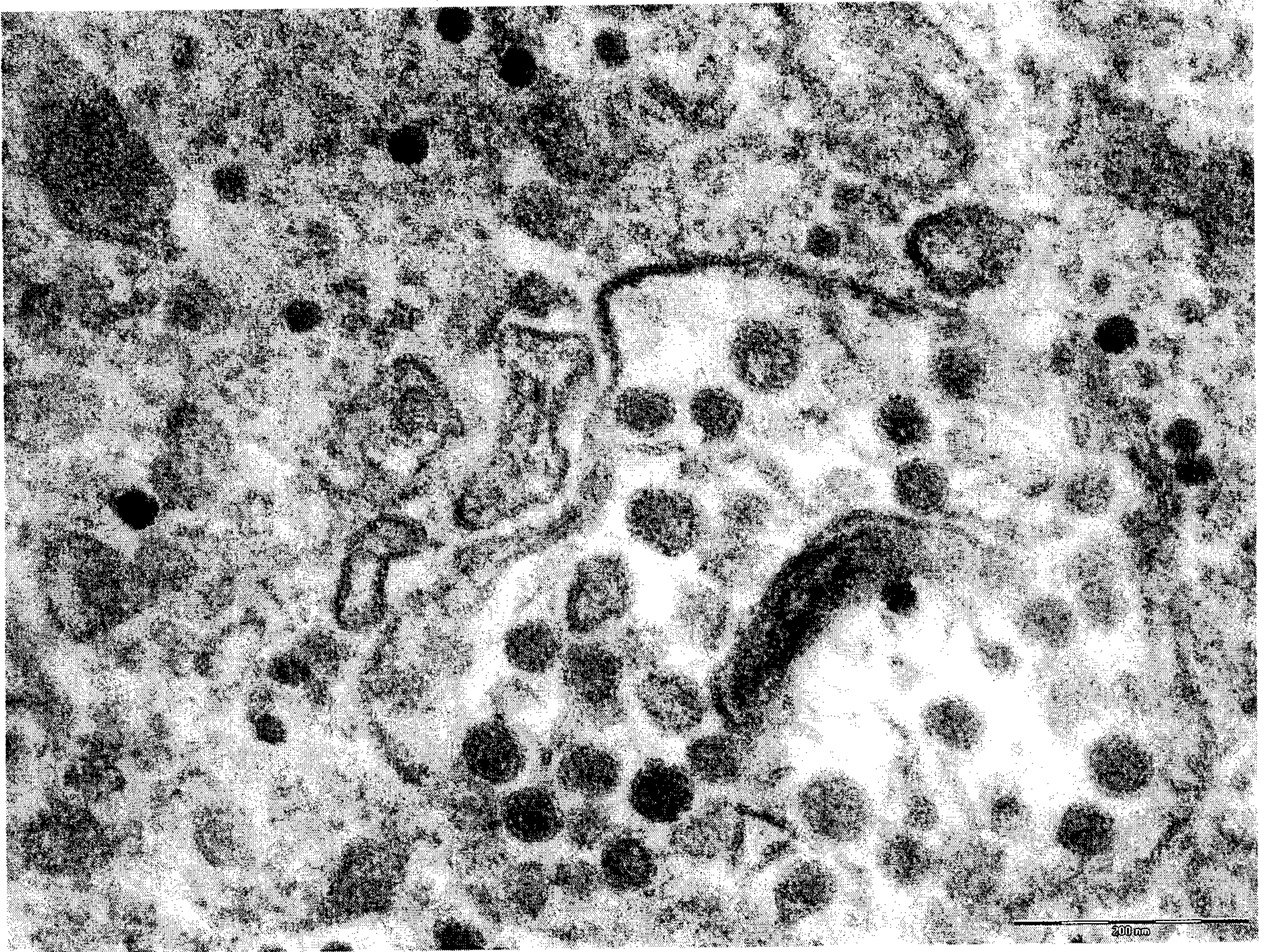


Fig. 9

