This is the peer reviewed version of the following article: *The sodium iodide symporter is unlikely to be a thyroid/breast shared antigen. Muller I, Zhang L, Giani C, Dayan CM, Ludgate ME, Grennan-Jones F. J Endocrinol Invest. 2016 Mar;39(3):323-31. doi: 10.1007/s40618-015-0368-6.*
The final publication is available at https://link.springer.com/article/10.1007/s40618-015-0368-6

The sodium iodide symporter is unlikely to be a thyroid/breast shared antigen

I. Muller[1], L. Zhang[1], C. Giani[2], C.M. Dayan[1], M.E. Ludgate[1] and F. Grennan-Jones[1]

(1) Thyroid Research Group, Institute of Molecular & Experimental Medicine, School of Medicine, Cardiff University, Cardiff, UK

(2) Department of Endocrinology, School of Medicine, University of Pisa, Italy

AUTHORS' CONTACT INFORMATION

Institute of Molecular & Experimental Medicine – Thyroid Research Group, Cardiff University - School of Medicine, University Hospital of Wales, Heath Park, Cardiff, UK, CF 14 4XN.

Dr. Ilaria Muller (MD) mulleri4@cardiff.ac.uk

Dr. Lei Zhang (PhD) zhangl14@cardiff.ac.uk

Prof. Dr. Colin Mark Dayan (PhD) dayancm@Cardiff.ac.uk

Prof. Marian Elizabeth Ludgate (PhD) ludgate@cardiff.ac.uk

Dr. Fiona Grennan-Jones (PhD) grennan-jonesfa@cardiff.ac.uk

Department of Endocrinology, University of Pisa, School of Medicine, Via Paradisa 2, 56124 Pisa, Italy.

Prof. Dr. Claudio Giani (MD) claudio.giani@med.unipi.it

CORRESPONDING AUTHOR

Dr. Ilaria Muller (MD), Clinical Research Fellow

Institute of Molecular & Experimental Medicine – Thyroid Research Group

Cardiff University - School of Medicine

University Hospital of Wales Main Building block C, 2nd floor, Room 256

Heath Park, Cardiff, UK, CF 14 4XN

Tel +44(0)2920742182 Fax +44(0)2920744671 mulleri4@cardiff.ac.uk

ABSTRACT

Purpose: Anti-thyroid peroxidase (TPO) autoantibodies (TPOAb) seem to be protective for patients with breast cancer (BC). Thyroid and breast tissues both express the sodium iodide symporter (NIS), similarly both have a peroxidase activity, TPO and lactoperoxidase (LPO) respectively. We hypothesize a common immune response to a thyroid/breast shared antigen suggesting 3 putative mechanisms: 1) TPOAb react to both TPO and LPO, 2) TPO could be expressed in BC and 3) patients with TPOAb could have autoantibodies to NIS (NISAb). Previous studies excluded NISAb that block NIS activity in sera of patients with thyroid autoimmunity (TA) and/or BC. This study investigates neutral NISAb (binding without affecting function).

Methods: Clones of CHO cells stably expressing human NIS (hNIS; CHO-NIS) were isolated following transfection of hNIS in pcDNA3 vector. Expression of hNIS mRNA and surface protein was confirmed by PCR and flow cytometry respectively using a hNIS-mouse-monoclonal-antibody. CHO-NIS and controls transfected with the empty pcDNA3 vector (CHO-Empty) were incubated with 42 heat-inactivated human sera followed by an anti-human-IgG-AlexaFluor488-conjugate: 12 with BC, 11 with TA, 10 with both BC and TA and 9 with non-autoimmune thyroid diseases. The Kolmogorov-Smirnov-Test was used to compare the fluorescence intensity obtained with CHO-NIS and CHO-Empty, using sera from 6 young males as a negative control population.

Results: None of the 42 sera were positive for NISAb.

Conclusions: NISAb are rare and NIS is unlikely to be a common thyroid/BC shared antigen. We have recently demonstrated TPO expression in BC tissue and are currently investigating TPOAb cross-reactivity with TPO/LPO.

KEYWORDS:

Thyroid autoimmunity, Sodium Iodide Symporter (NIS), Breast Cancer, Autoantibodies

INTRODUCTION

The relationship between breast cancer (BC) and benign thyroid disorders, especially thyroid autoimmunity (TA), has been widely reported over many years [1-3], but remains controversial [4-8]. In particular BC patients have been reported to have a higher prevalence of anti-thyroid peroxidase (TPO) autoantibodies (TPOAb) [6,9,10] with several authors reporting that TPOAb positive BC patients have a better outcome [11-14].

These observations have led us and others to propose an immune response to a shared thyroid/breast auto-antigen, which confers improved outcome in terms of breast tumor growth. Thyroid and breast tissue share several proteins having similar function. These include the sodium iodide symporter (NIS) [15,16] and enzymes with peroxidase activity, TPO and lactoperoxidase (LPO) respectively [17], from which we suggest 3 possible mechanisms for the common immune response. 1) TPO is present in both thyroid and BC cells and we recently demonstrated that TPO (both mRNA and protein) is expressed in BC tissue [18], even if at a much lower level compared with thyroid tissue. 2) TPOAb could cross react with LPO expressed in BC tissue, since TPO and LPO have a 46% primary sequence homology [19]. 3) The present study focuses on a third hypothesis: since NIS is expressed in thyroid and BC cells, TPOAb positive patients could also have autoantibodies to NIS (NISAb) reacting to NIS in both tissues, following spread of the autoimmune response.

The Human NIS (hNIS) gene encodes a 643 amino acid membrane-bound glycoprotein located at the baso-lateral portion of the thyroid follicular cell [15,20]. NIS has been detected in lactating breast tissue and the upregulation of NIS expression has been demonstrated in BC tissue by immunochemistry [16].

In 1995 Raspé et al. identified NIS as a potential thyroid autoantigen, albeit in a very small proportion of patients [21], while Kilbane et al. [22] reported low iodine content in BC tissue compared to benign breast tumours, postulating that putative NISAb were able to block hNIS function in BC cells. This was disputed in a study by Fierabracci et al. [23] who reported no interference of purified serum IgGs from patients with BC and TA on the iodine uptake by a Chinese hamster ovary (CHO) cell line stably transfected with hNIS.

A number of studies investigated the prevalence of NISAb using recombinant hNIS protein produced in an *in vitro* transcription/translation system. They used immunoprecipitation, SDS-PAGE and autoradiography to detect neutral NISAb (bind but do not affect NIS function), reporting their

presence in 24% [24], 20.8% [25] and 14% [26] of autoimmune hypothyroid patients, significantly higher than healthy control subjects in all cases. They also explored NISAb function with iodide uptake assays using CHO cells transfected with full-length hNIS and found the presence of NIS inhibiting activity in both sera and isolated IgGs, even if in 50% of inhibiting sera no antibodies binding NIS were found [24].

The aim of this present study is to evaluate the prevalence of neutral NISAb in patients affected by TA and/or BC. The presence of these antibodies, especially in BC patients, would support our hypothesis that NIS is a thyroid/breast-tissue shared auto-antigen.

MATERIALS AND METHODS

Establishment of CHO cell lines expressing hNIS

The hNIS complementary DNA (cDNA) cloned into the eukaryotic expression vector pcDNA3 and encoding amino acid 1 to 612 of hNIS was a gift from Dr. S.M. Jhiang (The Ohio State University, Columbus, Ohio) [27,28].

CHO cells were maintained in Ham's F-12 supplemented with 10% FCS, 2% penicillin/streptomycin, 1 mM sodium pyruvate and 0.1% sodium bicarbonate. CHO cells were transfected using TransFast® (Promega) according to the manufacturer's instructions. CHO cells transfected with pcDNA3-hNIS (CHO-NIS) and empty pcDNA3 vector (CHO-Empty) as a control were selected using G418. Positive CHO-NIS expressing clones were isolated by serial dilution.

NIS mRNA expression was tested in CHO-NIS clones and CHO-Empty controls; thyroid tissue obtained with ethical approval and informed consent from a patient undergoing thyroidectomy for Graves' disease served as a positive control. RNA extraction and reverse transcription were followed by polymerase chain reaction (35 cycles) and agarose gel analysis as previously described [18], amplifying a hNIS product of 207 bp using Forward Primer CTCCCTGCTAACGACTCCAG (1842-1861 exon 12) and Reverse Primer GAGGTCCCACCACAACAATC (2029-2048 exon 14) designed with Primer 3 Plus software.

We tested NIS protein expression on the surface of CHO-NIS cells by flow cytometry using a mouse monoclonal antibody to hNIS, as described below.

Flow cytometric analysis

CHO cells 70-90% confluent were detached from 75-cm^2 flasks with 5 ml PBS containing 5 mM EDTA/EGTA, transferred to universal tubes, and washed in PBS/0.5% BSA/2mM EDTA/20 mM Hepes (Flow Buffer). Cells were pelleted at 1200 RPM for 6.5 min at 4°C and the supernatant removed by inversion. Cells were counted and transferred to BD Falcon round bottom polystyrene tubes (Scientific laboratories supplies) and adjusted to 50,000 cells/tube.

Incubations with Flow Buffer (100µl) containing heat inactivated human serum from various patients (1:50) or the mouse monoclonal antibody VJ2 directed against an extracellular domain of hNIS (1:50, kindly donated by Dr S. Costagliola, Free University of Brussels) [29] were for 1 hour at room temperature.

Cells were then washed X3 with Flow Buffer (1ml) and centrifuged as above, followed by a 30 min incubation with Flow Buffer (100µl) containing goat polyclonal anti-human IgG (H+L) Alexa Fluor 488 (1:50, Life Technologies) or ab150113 goat polyclonal anti-mouse IgG H&L Alexa Fluor 488 (1:2000, abcam) respectively at 4°C in the dark.

A viability dye, LIVE/DEAD® Fixable Near-IR Dead Cell Stain Kit (50µl, 1:1000, Invitrogen) was added finally to each tube and incubated at 4°C in the dark for a further 30 min, to facilitate detection of damaged cells. Experimental controls omitting the first antibody (either hNIS monoclonal antibody or patients' serum) were also analyzed in the same experiment to investigate nonspecific binding of either mouse/ human Alexa Fluor conjugates.

Following the final incubation cells were washed X3 with Flow Buffer as before and re-suspended in 250µl Flow Buffer. The fluorescence of 10,000 cells/tube was assayed by BD FACSCanto II flow cytofluorometer, FACSDiva Software (BD Biosciences, San Jose, USA). FITC and Apc-Cy7 channels were used to detect Alexa Fluor 488 and Near-IR dead cell stain signals respectively. No compensation was needed since their excitation-emission peaks had different wavelengths (500-520 nm and 633-750 nm respectively).

Human sera used for flow cytometry

Sera from 42 women and negative control sera from 6 young males (Group M) were tested after heat inactivation at 56°C for 30 min. Patient were divided into 4 groups and their characteristics

are shown in Table 1. TPOAb and anti-thyroglobulin autoantibodies (TgAb) were measured by immunoenzymometric assay (Tosoh Bioscience, Tessenderlo, Belgium).

Flow cytometry with extracted immunoglobulins (Igs)

Igs were extracted from selected sera representing each patient group, incubating the sera at 4°C for 4 hrs with ammonium sulphate 3.75M (1.49 ml for each 1 ml serum) and then dialyzing X3 in PBS for 4 hrs [30].

Pre-adsorption of individual human sera using CHO-Empty cells

Selected sera representing each patient group were pre-incubated with CHO-Empty cells in a pre-adsorption step before incubation with CHO-NIS 6 and CHO-Empty cells for flow cytometry analysis. In brief, sera to be tested were diluted 1:10 with CHO-Empty cells re-suspended in 0.18mls of flow buffer and mixed gently for 1 hour at room temperature. Cells were removed by centrifugation, the pre-adsorbed sera recovered and used as described above in subsequent steps [31].

Permeabilization protocol for flow cytometry

Selected sera representing each patient group were tested by flow cytometry following a modified protocol in order to expose hypothetical internal NIS antigens through cell membrane permeabilization, which may hypothetically arise during the autoimmune destruction of thyroid tissue. Just before the incubation with the first antibody, CHO cells were fixed in 2% paraformaldehyde in PBS on ice for 30 min, washed x1 in PBS/0.1% BSA and then incubated with 0.1% BSA, 0.2% saponin PBS for 30 min at room temperature. The subsequent steps were similar to the original protocol, except they were all performed in 0.1% BSA, 0.2% saponin PBS instead of Flow buffer.

Computer data analysis

Flow Cytometric data was analyzed using FlowJo 8.8.6 Software (TreeStar Inc., Ashland, USA).

Damaged and dead cells (positive for Near-IR dead cell stain measured with Apc-Cy7 channel) were gated and excluded from the analysis. FITC channel fluorescence intensity values, relative to Alexa Fluor dye, were measured in CHO-NIS cells and compared with control CHO-Empty cells.

Statistical analysis

The geometric mean FITC fluorescence intensity values of CHO-NIS and CHO-Empty were compared for all sera and the Kolmogorov-Smirnov univariate two-sample test was used to obtain the greatest difference between the two histograms (CHO-NIS and CHO Empty), quoted as D value (D) [32]. Cut-off values were defined based on the mean D + 2 SD of the control young males sera with all values higher than this considered positive.

RESULTS

Characterization of CHO-NIS cell lines

Successful CHO-NIS transfections were confirmed by the presence of a 207 bp amplicon in a mixed pool of CHO-NIS transfected cells; no amplicons were obtained in CHO-Empty (Fig. 1 panel A). We also tested 6 individual CHO-NIS clones, but only Clones 1 and 6 (CHO-NIS 1 and CHO-NIS 6) expressed NIS mRNA (images not shown).

Flow cytometric analysis using VJ2 confirmed a positive signal with CHO-NIS 6 and CHO-NIS 1 cells compared to CHO-Empty (10^4, 10^3 and 10^2 mean fluorescent signal intensity respectively), indicating high specific binding of VJ2 to the NIS protein expressed on the cells surface, with CHO-NIS 6 expressing the NIS protein at a higher level than CHO-NIS 1 (Fig. 1 panels B and C).

Flow cytometry with human sera

All 42 sera were tested in 3 separate experiments: once with CHO-NIS 1 and twice using CHO-NIS 6, always compared to CHO-Empty as negative control and testing Group M as reference control.

In the three experiments all 42 human sera were negative for NISAb, i.e. no sera had a D value higher than the mean D + 2 SD of Group M. There were some borderline positive sera similarly distributed in all 4 groups which had D values close to the D + 2 SD cut-off of Group M. Representative flow cytometry histograms are shown in Fig. 2 panels A and B, representing borderline positive and negative sera respectively.

Flow cytometry performed using human sera, but not VJ2, showed a high non-specific signal with both CHO-NIS clones (1&6) and CHO-Empty cells. This high background was absent when the same cells were incubated with the second anti-human conjugate antibody only (Fig. 2 panel C), suggesting that the presence of this nonspecific signal is due to the human sera itself.

To clarify this issue we performed some flow cytometry experiments with extracted Igs from 4 representative sera (one for each patient group) that showed the same nonspecific background with both CHO-NIS and CHO-Empty cells obtained when using human sera (images not shown), suggesting the presence of serum antibodies against unknown antigens expressed on the CHO cells surface.

We then carried out a pre-absorption protocol in which human sera were incubated with CHO-Empty cells to try and eliminate or reduce the nonspecific binding. As shown in Fig. 3, pre-adsorption was effective in reducing the nonspecific background in 6/7 (85.7%) human sera for both CHO-Empty and CHO-NIS 6 cells. Contrary to our expectations, the pre-adsorption step was unable to significantly enhance any difference between CHO-Empty and CHO-NIS 6 cells (signal specificity), since it was only minimally increased in 4/7 (57%) (panels A and B), minimally decreased in 2/7 (29%) (panels C and D) and unchanged in 1/7 (14%) (panels E and F). Therefore we decided to perform screening of the 42 human sera without this pre-adsorption step.

The experiment testing 7 different human sera with permeabilized CHO-NIS and CHO-Empty cells demonstrated a further increase in nonspecific background in both CHO-NIS and CHO-Empty cells without any significant difference between cells expressing NIS and controls (Fig. 4).

DISCUSSION

In this study none of the 42 human sera examined were positive for neutral NISAb, as no signals above the positive cut-off value were found. However 8/42 sera (19%), distributed throughout the 4 patient groups, were considered borderline positive, since they fell near to the positive cut-off. These higher D values, even if not statistically significant, could be representative of low titre neutral NISAb and/or demonstrate random variability without any significance. We are more inclined to the second interpretation as these 8 borderline positive sera were not confirmed in all three experiments performed. We therefore conclude that only D values clearly above the cut-off should be considered positive and we did not find any positive sera in this study.

To our knowledge this is the first paper using flow cytometry to detect neutral serum NISAb binding the symporter. Previously other groups using a recombinant hNIS protein identified neutral NISAb in sera from patients affected by TA, but which were also present in the general population [24-26]. Our results do not confirm these previous reports. The most likely explanation is that expression of hNIS using an *in vitro* transcription/translation system may result in different and/or incomplete folding of the hNIS protein and a different glycosylation pattern compared with the recombinant hNIS produced in our CHO cell system. We believe that CHO expression of this complex protein with its 13 transmembrane domains allows complete folding and glycosylation of this iodide transporter, with expression in its original location as a transmembrane protein. It is therefore probable that the conformational hNIS epitopes recognized by antibodies are likely to be different between the two recombinant protein expression techniques.

We can conclude from this investigation that serum neutral NISAb are rare, in agreement with a number of previous studies [21,33,34] although not all [24,28]. Similarly, NISAb interfering with the symporter function have been described as rare by several authors who found no iodide uptake inhibitory activity among extracted IgGs from patients with or without TA and hypothesized that the inhibitory activity observed in some sera was no antibody-mediated, since disappearing when using IgGs instead of sera [23,33,34]. On the contrary, Ajjan et al. found iodide uptake inhibitory activity mediated by IgGs, even if 50% of those IgGs did not bind NIS, therefore they also postulated that the inhibitory activity could be in part not antibody-mediated or due to antibodies that do not act directly on NIS [24,28].

We conclude that NIS is unlikely to be a shared antigen responsible for a common immune response between thyroid and BC tissue. However, to definitively address this question further studies with larger number of sera are needed. In addition alternative techniques such as ELISA or Western Blot could be used to detect NISAb [35], as such techniques may expose epitopes of the hNIS protein not accessible when anchored in the cell membrane. Furthermore, differences in protein folding are possible with these techniques which again may result in a greater diversity of epitope exposure. In this study we cannot exclude the presence of NISAb recognizing intracellular epitopes which may arise following cell and tissue damage and which would be inaccessible in the flow protocol applied. Our preliminary experiments using permeabilized cells were hampered by high non-specific background staining which precluded our drawing any conclusions. This problem has been reported by other

authors using flow cytometry to detect antibodies to the thyrotropin receptor (TSHR) [31,36]. Since the background remains even when using purified Igs and is only slightly reduced by pre-adsorption, it is clearly due to antibodies to surface CHO proteins. CHO cells are the most commonly used mammalian cell lines for production of therapeutics [37-39] and some 60-70% of recombinant protein pharmaceuticals and almost all currently approved therapeutic antibodies are produced in mammalian cells [40]. This would account for some CHO-reactivity [41,42] but Xue et al. analyzed 83 normal individuals with no known exposure to therapeutic biologics and found antibodies to CHO proteins in 45/83 (54%) cases, mainly isotype G Igs (IgGs). Therefore they postulated alternative explanations such as blood transfusions, vaccinations or keeping animals as pets [43].

Despite this problem we have confidence in the flow cytometry protocol used, which has previously served to demonstrate neutral antibodies to another thyroid autoantigen, the TSHR [31,36,44].

In conclusion no neutral NISAb were found in 42 human sera from different patients with TA and/or BC. The results confirm those of other groups who found NISAb blocking NIS activity only in a very small proportion of tested subjects [21,23,33,34]. Therefore NIS is unlikely to be a putative shared antigen between thyroid and BC cells, responsible for suggested improved outcomes of BC in patients affected by TA, even if further experiments are needed to definitively address this question, e.g. searching for internal epitopes potentially arising only during or after tissue damaging. Furthermore, the absence of NISAb does not exclude that NIS linear epitopes could trigger a hypothetical lymphocytes T reaction and specific studies are needed to explore this possibility. However one of the hypothetical common antigens is likely to be TPO, since it is expressed in both thyroid and BC tissue [18] and some studies suggest that BC patients positive for TPOAb have a better prognosis than patients negative for TPOAb [11-14]. Cross-reactivity between TPOAb and LPO has also been described [45,46] and further investigations are required to clarify the thyroid/breast shared antigen.

ACKNOWLEDGMENTS

This project has been supported by the "2013 Tenovus Innovation Grant" of Tenovus Cancer Care (Cardiff, Wales, UK) to Dr. Ilaria Muller.

The authors are also grateful to Dr. P.A. Smanik and Dr. S.M. Jhiang (Department of Physiology and Department of Internal Medicine, The Ohio State University, Columbus, Ohio) for the provision of

hNIS cDNA and to Dr S. Costagliola [IRIBHM (Institute of Interdisciplinary Research in Molecular Human Biology), Université Libre de Bruxelles, Brussels, Belgium] for the gift of the NIS antibody VJ2.

AUTHOR DISCLOSURE STATEMENT

The authors declare that no conflicts of interest exist.

REFERENCES

1. Loeser AA (1954) A new therapy for prevention of post-operative recurrences in genital and breast cancer; a six-years study of prophylactic thyroid treatment. Br Med J 2:1380-3
2. Chalstrey LJ, Benjamin B (1966) High incidence of breast cancer in thyroid cancer patients. Br J Cancer 20:670-5
3. Mittra I, Hayward JL (1974) Hypothalamic-pituitary-thyroid axis in breast cancer. Lancet 1:885-9
4. Maruchi N, Annegers JF, Kurland LT (1976) Hashimoto's thyroiditis and breast cancer. Mayo Clin Proc 51:263-5
5. Hedley AJ, Spiegelhalter DJ, Jones SJ, Clements P (1981) Thyroid disease and breast cancer. Lancet 1:558
6. Giani C, Fierabracci P, Bonacci R, Gigliotti A, Campani D, De Negri F, Cecchetti D, Martino E, Pinchera A (1996) Relationship between breast cancer and thyroid disease: relevance of autoimmune thyroid disorders in breast malignancy. J Clin Endocrinol Metab 81:990-4
7. Sarlis NJ, Gourgiotis L, Pucino F, Tolis GJ (2002) Lack of association between Hashimoto thyroiditis and breast cancer: a quantitative research synthesis. Hormones (Athens) 1:35-41
8. Michalaki V, Kondi-Pafiti A, Gennatas S, Antoniou A, Primetis H, Gennatas C (2009) Breast cancer in association with thyroid disorders. J BUON 14:425-8
9. Gogas J, Kouskos E, Tseleni-Balafouta S, Markopoulos C, Revenas K, Gogas G, Kostakis A (2001) Autoimmune thyroid disease in women with breast carcinoma. Eur J Surg Oncol 27:626-30
10. Giustarini E, Pinchera A, Fierabracci P, Roncella M, Fustaino L, Mammoli C, Giani C (2006) Thyroid autoimmunity in patients with malignant and benign breast diseases before surgery. Eur J Endocrinol 154:645-9
11. Smyth PP, Shering SG, Kilbane MT, Murray MJ, McDermott EW, Smith DF, O'Higgins NJ (1998) Serum thyroid peroxidase autoantibodies, thyroid volume, and outcome in breast carcinoma. J Clin Endocrinol Metab 83:2711-6
12. Fiore E, Giustarini E, Mammoli C, Fragomeni F, Campani D, Muller I, Pinchera A, Giani C (2007) Favorable predictive value of thyroid autoimmunity in high aggressive breast cancer. J Endocrinol Invest 30:734-8
13. Farahati J, Roggenbuck D, Gilman E, Schutte M, Jagminaite E, Seyed Zakavi R, Loning T, Heissen E (2012) Anti-thyroid peroxidase antibodies are associated with the absence of distant metastases in patients with newly diagnosed breast cancer. Clin Chem Lab Med 50:709-14
14. Prinzi N, Baldini E, Sorrenti S, De Vito C, Tuccilli C, Catania A, Carbotta S, Mocini R, Coccaro C, Nesca A, Bianchini M, De Antoni E, D'Armiento M, Ulisse S (2014) Prevalence of breast cancer in thyroid diseases: results of a cross-sectional study of 3,921 patients. Breast Cancer Res Treat
15. Carrasco N (1993) Iodide transport in the thyroid gland. Biochim Biophys Acta 1154:65-82
16. Tazebay UH, Wapnir IL, Levy O, Dohan O, Zuckier LS, Zhao QH, Deng HF, Amenta PS, Fineberg S, Pestell RG, Carrasco N (2000) The mammary gland iodide transporter is expressed during lactation and in breast cancer. Nat Med 6:871-8
17. O'Brien PJ (2000) Peroxidases. Chem Biol Interact 129:113-39
18. Muller I, Giani C, Zhang L, Grennan-Jones FA, Fiore E, Belardi V, Rosellini V, Funel N, Campani D, Giustarini E, Lewis MD, Bakhsh AD, Roncella M, Ghilli M, Vitti P, Dayan CM,

Ludgate ME (2014) Does thyroid peroxidase provide an antigenic link between thyroid autoimmunity and breast cancer? Int J Cancer 134:1706-14

19. Sharma S, Singh AK, Kaushik S, Sinha M, Singh RP, Sharma P, Sirohi H, Kaur P, Singh TP (2013) Lactoperoxidase: structural insights into the function,ligand binding and inhibition. Int J Biochem Mol Biol 4:108-128

20. Dai G, Levy O, Carrasco N (1996) Cloning and characterization of the thyroid iodide transporter. Nature 379:458-60

21. Raspe E, Costagliola S, Ruf J, Mariotti S, Dumont JE, Ludgate M (1995) Identification of the thyroid Na+/I- cotransporter as a potential autoantigen in thyroid autoimmune disease. Eur J Endocrinol 132:399-405

22. Kilbane MT, Ajjan RA, Weetman AP, Dwyer R, McDermott EW, O'Higgins NJ, Smyth PP (2000) Tissue iodine content and serum-mediated 125I uptake-blocking activity in breast cancer. J Clin Endocrinol Metab 85:1245-50

23. Fierabracci P, Pinchera A, Tonacchera M, Agretti P, De Marco G, Albertini S, Conforti G, Seregni E, Agresti R, Grasso L, Giani C (2004) Absence of interference of serum IgGs from patients with breast cancer and thyroid autoimmunity on the function of human iodide symporter gene stably transfected in CHO cells. J Endocrinol Invest 27:862-5

24. Ajjan RA, Kemp EH, Waterman EA, Watson PF, Endo T, Onaya T, Weetman AP (2000) Detection of binding and blocking autoantibodies to the human sodium-iodide symporter in patients with autoimmune thyroid disease. J Clin Endocrinol Metab 85:2020-7

25. Seissler J, Wagner S, Schott M, Lettmann M, Feldkamp J, Scherbaum WA, Morgenthaler NG (2000) Low frequency of autoantibodies to the human Na(+)/I(-) symporter in patients with autoimmune thyroid disease. J Clin Endocrinol Metab 85:4630-4

26. Brix TH, Hegedus L, Weetman AP, Kemp HE (2014) Pendrin and NIS antibodies are absent in healthy individuals and are rare in autoimmune thyroid disease: evidence from a Danish twin study. Clin Endocrinol (Oxf)

27. Smanik PA, Liu Q, Furminger TL, Ryu K, Xing S, Mazzaferri EL, Jhiang SM (1996) Cloning of the human sodium lodide symporter. Biochem Biophys Res Commun 226:339-45

28. Ajjan RA, Findlay C, Metcalfe RA, Watson PF, Crisp M, Ludgate M, Weetman AP (1998) The modulation of the human sodium iodide symporter activity by Graves' disease sera. J Clin Endocrinol Metab 83:1217-21

29. Pohlenz J, Duprez L, Weiss RE, Vassart G, Refetoff S, Costagliola S (2000) Failure of membrane targeting causes the functional defect of two mutant sodium iodide symporters. J Clin Endocrinol Metab 85:2366-9

30. Liddell J (2003) Antibody Purification by Ammonium Sulfate Precipitation. eLS. John Wiley & Sons Ltd, Chichester. http://www.els.net

31. Jaume JC, Kakinuma A, Chazenbalk GD, Rapoport B, McLachlan SM (1997) Thyrotropin receptor autoantibodies in serum are present at much lower levels than thyroid peroxidase autoantibodies: analysis by flow cytometry. J Clin Endocrinol Metab 82:500-7

32. Young IT (1977) Proof without prejudice: use of the Kolmogorov-Smirnov test for the analysis of histograms from flow systems and other sources. J Histochem Cytochem 25:935-41

33. Chin HS, Chin DK, Morgenthaler NG, Vassart G, Costagliola S (2000) Rarity of anti- Na+/I- symporter (NIS) antibody with iodide uptake inhibiting activity in autoimmune thyroid diseases (AITD). J Clin Endocrinol Metab 85:3937-40

34. Tonacchera M, Agretti P, Ceccarini G, Lenza R, Refetoff S, Santini F, Pinchera A, Chiovato L, Vitti P (2001) Autoantibodies from patients with autoimmune thyroid disease do not interfere with the activity of the human iodide symporter gene stably transfected in CHO cells. Eur J Endocrinol 144:611-8

35. Kucharska AM, Czarnocka B, Demkow U (2013) Anti-natrium/iodide symporter antibodies and other anti-thyroid antibodies in children with Turner's syndrome. Adv Exp Med Biol 756:131-8

36. Patibandla SA, Dallas JS, Seetharamaiah GS, Tahara K, Kohn LD, Prabhakar BS (1997) Flow cytometric analyses of antibody binding to Chinese hamster ovary cells expressing human thyrotropin receptor. J Clin Endocrinol Metab 82:1885-93

37. Jones D, Kroos N, Anema R, van Montfort B, Vooys A, van der Kraats S, van der Helm E, Smits S, Schouten J, Brouwer K, Lagerwerf F, van Berkel P, Opstelten DJ, Logtenberg T, Bout A (2003) High-level expression of recombinant IgG in the human cell line per.c6. Biotechnol Prog 19:163-8

38. Werner RG, Noe W, Kopp K, Schluter M (1998) Appropriate mammalian expression systems for biopharmaceuticals. Arzneimittelforschung 48:870-80

39. Wurm FM (2004) Production of recombinant protein therapeutics in cultivated mammalian cells. Nat Biotechnol 22:1393-8
40. Schirrmann T, Al-Halabi L, Dubel S, Hust M (2008) Production systems for recombinant antibodies. Front Biosci 13:4576-94
41. Koren E, Zuckerman LA, Mire-Sluis AR (2002) Immune responses to therapeutic proteins in humans--clinical significance, assessment and prediction. Curr Pharm Biotechnol 3:349-60
42. Shankar G, Pendley C, Stein KE (2007) A risk-based bioanalytical strategy for the assessment of antibody immune responses against biological drugs. Nat Biotechnol 25:555-61
43. Xue L, Johnson R, Gorovits B (2010) Prevalence and isotypic complexity of the anti-Chinese hamster ovary host cell protein antibodies in normal human serum. AAPS J 12:98-106
44. Metcalfe R, Jordan N, Watson P, Gullu S, Wiltshire M, Crisp M, Evans C, Weetman A, Ludgate M (2002) Demonstration of immunoglobulin G, A, and E autoantibodies to the human thyrotropin receptor using flow cytometry. J Clin Endocrinol Metab 87:1754-61
45. Banga JP, Tomlinson RW, Doble N, Odell E, McGregor AM (1989) Thyroid microsomal/thyroid peroxidase autoantibodies show discrete patterns of cross-reactivity to myeloperoxidase, lactoperoxidase and horseradish peroxidase. Immunology 67:197-204
46. Ruf J, Toubert ME, Czarnocka B, Durand-Gorde JM, Ferrand M, Carayon P (1989) Relationship between immunological structure and biochemical properties of human thyroid peroxidase. Endocrinology 125:1211-8

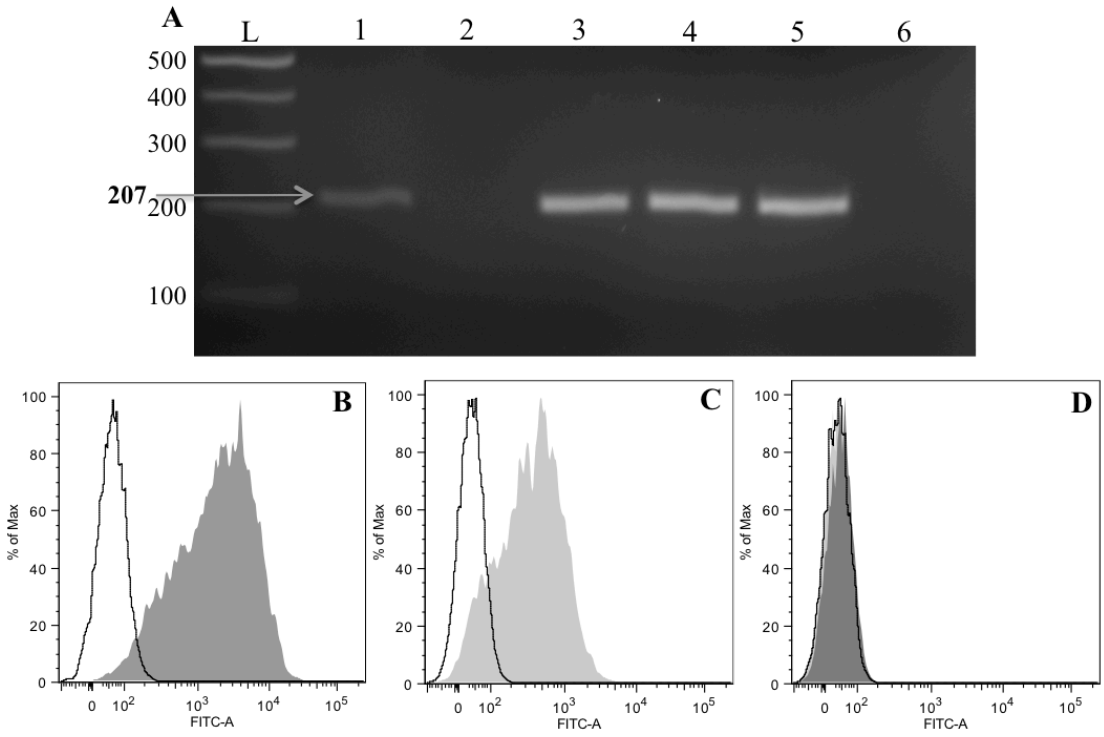
TABLES AND FIGURES

Table 1. Human sera

	Age: Mean and Range	TPOAb positive	TgAb positive
BC (n=12)	66 y ± 12 y SD (range 42-81 y)	0/12	1/12
TA (n=11)	48 y ± 13 y SD (range 31-76 y)	10/11	9/11
BC/TA (n=10)	54 y ± 7 y SD (range 43–63 y)	9/10	8/10
C (n=9)	51 y ± 21 y SD (range 13-68 y)	0/9	1/9
M (n=6)	10 y, 3.3 m ± 5 m SD (range 9y, 6m – 10y, 6m)	0/6	0/6

Characteristics of subjects whose sera were tested using flow cytometry. BC = patients with breast cancer. TA = patients with thyroid autoimmunity (defined as serum TPOAb and/or TgAb positivity). BC/TA = patients with BC and TA. C = patients affected by non-autoimmune thyroid disorders. M = young males sera (control population). y = years. m = months. SD = standard deviation. TPOAb = autoantibodies to thyroid peroxidase. TgAb = autoantibodies to thyroglobulin.

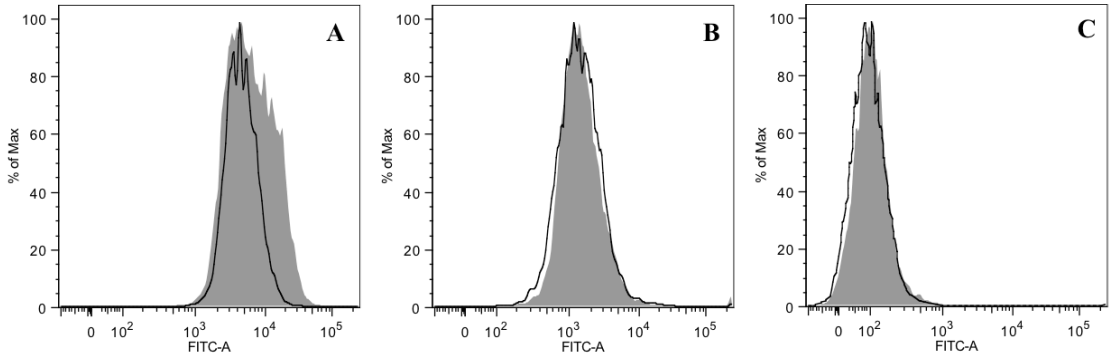
Fig. 1. Testing CHO-NIS cells for hNIS mRNA and protein expression



Panel A. PCR amplicons obtained with NIS primers after 35 cycles. 1 = thyroid tissue (positive control); 2 = CHO-Empty; 3, 4, 5 = CHO-NIS 1st, 2nd and 3rd transfection respectively; 6 = distilled water (negative control). The 207bp expected amplicon is observed in 1, 3, 4, 5 and absent in 2, 6.

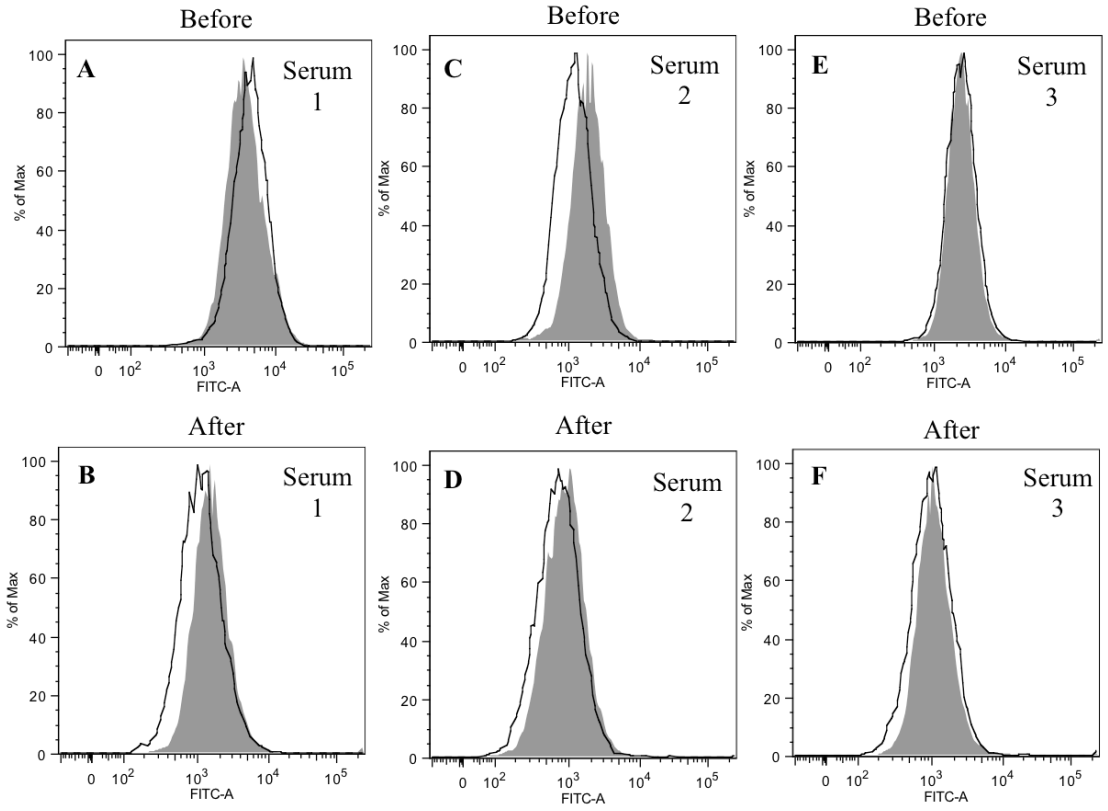
Panels B,C,D. Flow cytometry histograms showing the FITC fluorescence intensity using VJ2 with second anti-mouse IgGs conjugate antibody in CHO-NIS 6 (panel B; dark grey tinted figure; 10^4) and CHO-NIS 1 (panel C; light grey tinted figure; 10^3) compared with CHO-Empty (empty black line; 10^2). Panel D shows incubation with the second anti-mouse IgGs antibody only (without VJ2 incubation): the signal is negative (10^2) in all three cell clones, excluding aspecific binding of the second antibody.

Fig. 2. Flow cytometry results with human sera



Representative flow cytometry histograms showing the FITC fluorescence intensity obtained in CHO-Empty (empty black line) and CHO-NIS 6 (dark grey tinted figure) after incubation with human sera and second anti-human IgGs antibody conjugate. Panels A and B show borderline positive and negative results respectively, in presence of a high aspecific background signal (10^3-10^4). Panel C shows incubation with the second anti-human IgGs antibody conjugate only: the signal is negative (10^2) in both cell lines, indicating absence of nonspecific binding of the second antibody.

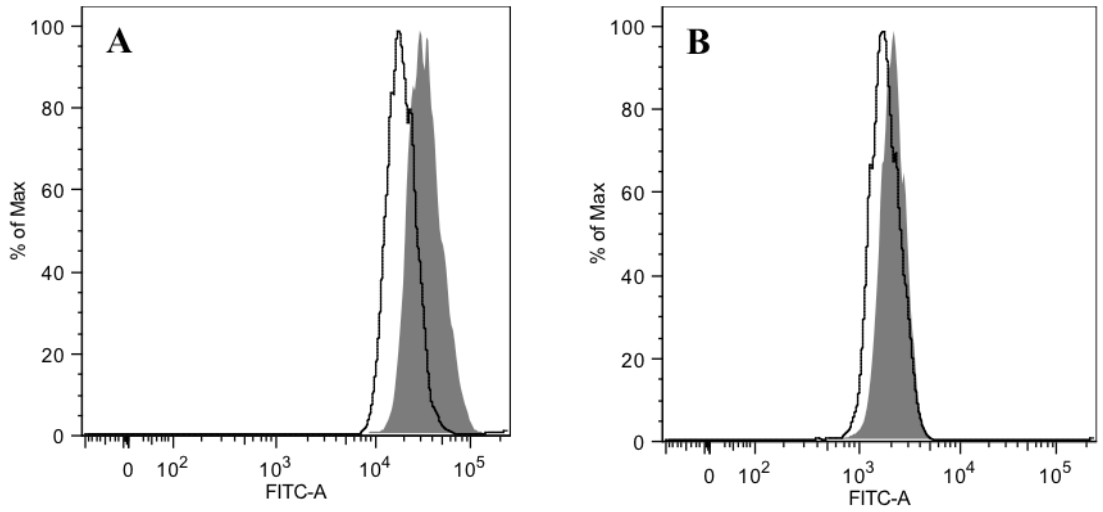
Fig. 3. Pre-adsorption step in flow cytometry



Representative flow cytometry histograms of FITC fluorescence intensity in CHO-Empty (empty black line) and CHO-NIS 6 (dark grey tinted figure) cell lines incubated with 3 human sera before (upper panels A, C, E) and after (lower panels B, D, F) adsorption with CHO-Empty cells. All panels show a shift of the histograms on the left for both CHO-Empty and CHO-NIS 6 after pre-adsorption step, indicating a nonspecific binding reduction.

The signal difference between CHO-Empty and CHO-NIS 6 cells after the pre-adsorption step in respect to before pre-adsorption is slightly increased in serum 1, slightly reduced in serum 2 and unchanged in serum 3.

Fig. 4. Flow cytometry with permeabilized cells



Representative flow cytometry histograms of FITC fluorescence intensity obtained using permeabilized CHO-Empty (empty black line) and CHO-NIS 6 (dark grey tinted figure). Panel A: results using human sera and second anti-human-IgG antibody conjugate. Panel B: negative control using second antibody only. In both cases there is a high nonspecific background signal present with both CHO-NIS and CHO-Empty cell lines, 10^4-10^5 and 10^3-10^4 respectively.