Diagnostic value of intraepithelial lymphocytes in celiac disease

Final thesis

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1. ABSTRACT

The main objective in the current work is to study the variation of the intraepithelial lymphocytes percentage between celiac and healthy patients. The flow cytometry has been used as a cell counting method. The most important results are that the IEL percentage is an appropriate validation method of flow cytometry in possible celiac patients and this can also be used as a biomarker parameter to diagnose celiac disease. The potential application of the IEL as a marker makes it a promising candidate in celiac disease diagnosis in the near future.

2. INTRODUCTION

2.1. Pathophysiology

Celiac disease (CD), sometimes called celiac sprue or gluten-sensitive enteropathy, is a disease that affects the small intestine by damaging its lining. It is a disease caused by a gluten hypersensitivity that causes the body's own antibodies to attack the villi of the small intestine.[1, 2, 3] The way the disease is affected is as follows:

When gluten is introduced into the body, the fractions of glutein protein (polymeric) and gliadin protein (monomeric) activate the innate and adaptive immune response of the patients with a genetic predisposition. This predisposition is caused by the DQ2 and DQ8 alleles.[3, 4, 5]

The gliadin protein has fragments that are formed by proline and glutamine, which confers resistance to degradation by intraluminal proteases and membrane enzymes. Therefore, the gluten fragments may end up piercing the paracellular and transcellular epithelium.[6, 7]

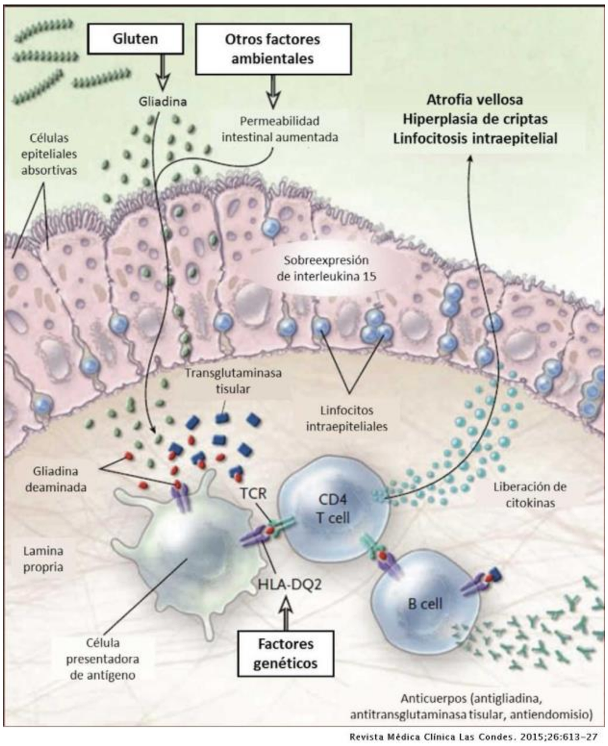


Figure 1: Pathophysiology of celiac disease.[8]

The paracellular pathway depends on the union of the epithelial cells, also known as permeability. This permeability is regulated by the zonulin peptide, for which it is believed that gluten is a stimulant of this protein, making the entrance of the gluten easier in the intraepithelial zone.[6]

The transcellular pathway depends on the Immunoglobulins A (IgA), which bind to gliadin are internalized by the transferrin receptor. Then, the tissue transglutaminase 2 (Ttg2), deamine gliadin, which becomes negatively charged ang binds to the domain of HLA DQ2/DQ8 of antigen-presenting cells, which triggers the immune response regulated by lymphocytes T CD4+.[6, 7]

Once the lymphocytes T are activated, they produce pro-inflammatory cytokines (TNFα, TNFγ), which activate the lymphocytes B response. These lymphocytes B generate antibodies against Ttg2 causing damage to the intestinal barrier and to the enterocytes or also known as intestinal absorptive cells. Innate immunity will generate a response producing interleukin-15, which regulates the IEL cytotoxicity. Once the intraepithelial lymphocytes are activated, they are responsible for the injuries caused in the intestinal epithelium.[6]

2.2. Diagnostic criteria

Several symptoms may indicate a celiac disease, diarrhoea, vomits, digestive problems are found among them. These symptoms are more frequent in patients that present classic celiac disease (normally in children). Instead, these symptoms may not appear in adults, which would cause a more difficult diagnosis of celiac disease like the cause of their problems.[6] There are four parameters to consider, apart from the symptoms, to diagnose a patient with celiac disease.[2, 9]

- Antibodies: the amount of antibodies is increased. As previously mentioned, celiac disease is an autoimmune disease, which means that our organism is attacking our own cells. The more frequent antibodies are the anti-transglutaminase and the antibodies anti-endomisio like a confirmatory test.

- Genetic tests: patients with celiac disease have been shown to be genetically predisposed. Two types of genes are known as responsible for this genetic predisposition, and both of them belong to the HLA (human lymphocyte antibodies) system. These genes are DQ2 present in 90% of patients and the other one is DQ8, present in 5% of patients.

- Biopsy: involves removing a sample of the duodenum and using a flow cytometry counting the different cells involved in the diagnosis of celiac disease. In this case these cells are the gamma delta positive intraepithelial lymphocytes and the CD3- lymphocytes. The current study will be focused on this analysis.

- Gluten-free diet: the patient must follow a very strict gluten-free diet for at least 6 months to confirm that has celiac disease. If there is an improvement in symptoms, it is considered an additional diagnostic parameter.

The four parameters mentioned above together with the symptoms defined make up the five different diagnostic tests to be done in patients. Patients with positive results in four of them are confirmed as celiac.[9]

The diagnosis in children is slightly different. Paediatric patients with genetic predisposition, only need two parameters to be celiac:

- The value of the antibodies anti-transglutaminase must be ten times higher than the value of reference in two different time-spaced analytics.

- The antibodies anti-endomysium test.

If these two parameters are positive, the patient will be diagnosed with celiac disease without requiring a biopsy. [10]

3. <u>GOALS</u>

The **main goal** of the thesis is: Study the percentage of intraepithelial lymphocytes (IEL) in possible celiac patients.

The **secondary goals** to study are the following:

- Learn to use flow cytometry as an analysis method.
- Validate the results of flow cytometry using the percentage of IEL.
- Use statistics values to validate or refuse a possible new biomarker.

4. MATERIAL AND METHODS

4.1. Patients

To perform this study, we worked with a group of 243 patients under 20 years and the samples were taken during the past five years (2017 to 2022). In the sample we observe a slightly larger population of women 56,79% in front of 43,21% of men. The age of the patients was distributed as follows: 31 between 0 and 5 years, 100 between 6 and 10 years, 96 between 11 and 15 years and finally 16 between 16 and 20 years.

4.2. Materials

- Flow cytometry "is a technology that rapidly analyses single cells or particles as they flow past single or multiple lasers while suspended in a buffered salt-based solution. Each particle is analysed for visible light scatter and one or multiple fluorescence parameters. Visible light scatter is measured in two different directions, the forward direction (Forward Scatter or FSC) which can indicate the relative size of the cell and at 90° (Side Scatter or SSC) which indicates the internal complexity or granularity of the cell. Light scatter is independent of fluorescence. Samples are prepared for fluorescence measurement through transfection and expression of fluorescent proteins, staining with fluorescent dyes or staining with fluorescently conjugated antibodies".[11]

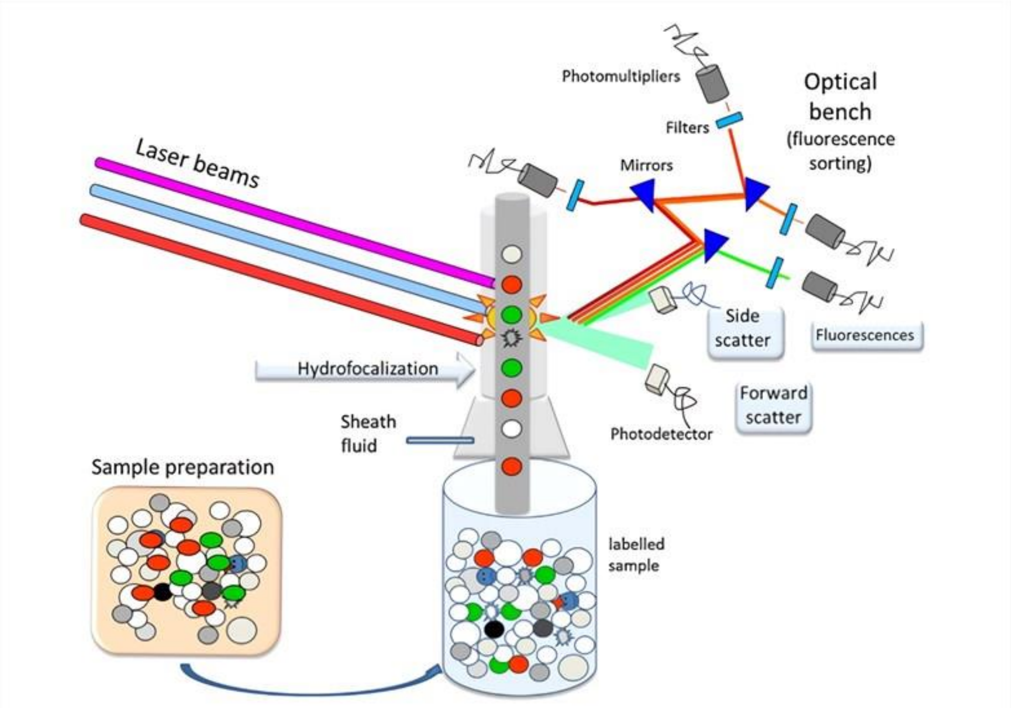


Figure 2: Internal functioning of the flow cytometer. [12]

- Calibrators (cytometer):
    - APC calibrate Ref. 340487. BD
    - Calibrate 3 color kit (PerCP, PE, FITC). Ref. 340486. BD.
    - Calibrate HLA B27. REF. 340183. BD

- Cell Wash. Ref. 349524. BD.
- FACSClean. Ref. 340345. BD.
- FACSRinse. Ref. 340346. BD.
- FACSFLOW. Ref. 342003. BD.
- Medium RPMI 1640 (growth medium).
- Beef serum.
- FACS - CD103 FITC 50 TEST.
- FACS - ANTI-TCRgd PE 100 TEST.
- FACS - CD3 PerCP 100 TEST.
- FACS - CD45 APC 100 TEST.
- EDTA tripotassium dihydrate salt 250g.
- DTT (dithiothreitol) 5g.
- Vortex.

4.3. Getting samples

The samples are obtained from patients with symptomatology compatible with celiac disease such as abdominal pain, diarrhoea, inflammation.

The process for obtaining the samples is a duodenal biopsy. A part of the duodenum is extracted and it is preserved in a complete medium solution.

4.4. Preparation of samples

4.4.1. Reagents

- Preparation of complete medium solution: We need 10 mL of RPMI1640 medium and added 1mL of beef serum. It is necessary to prepare the solution the day that it is going to be used and it is stable for a maximum of 24 hours.
- EDTA 0,1M: Molar mass EDTA tripotassium dihydrate salt 442,56 g/mol.

$$100\ mL * \frac{1L}{1000mL} * \frac{0{,}1\ mol}{1L} * \frac{442{,}56g}{1\ mol} = 4{,}4256g$$

To prepare the solution we added 4,4256 grams of EDTA tripotassium dihydrated salt in 100 mL of water. Stable for 6 months.

- DTT 0,5M: Molar mass DTT 154,253 g/mol.

$$5\ mL * \frac{1L}{1000mL} * \frac{0{,}5\ mol}{1L} * \frac{154{,}253g}{1\ mol} = 0{,}385g$$

To prepare the solution we added 0,385 grams of DTT in 5 mL of water. The solutions will be frozen in vials of 100 µL at a temperature of -20ºC. Stable for 1 year.

4.4.2. Procedure

First of all, we need to do the **desepitelization**:

- Check for presence of the sample in the medium
- Add 20 µL DTT 0,5 M and 100 µL EDTA 0,1 M to the tube with the sample and the complete medium.
- Leave to stir in a vertical rotor at 12 rpm during 60 minutes.

After that, a **separation** is required:

- Withdraw the biopsy with an inoculation loop and save it in a tube with 500 µL in case the process needs to be repeated.
- Centrifuge the tube where we had performed the desepitelization process at 1800rpm during 7 minutes.
- Remove the supernatant by settling and the pellet is resuspended with 70 µL of CELLWASH.

4.4.3. Marking

To separate the cells with a study interest, we need to add markers to group them. Prepare a tube of cytometry for each sample with 70 µL of precipitate. Add at each sample the volume of monoclonal antibodies following Table 1.

Table 1: Volume necessary of the different monoclonal antibodies.

Markers	Volume (µL)
PE TCRgd	10
FITC CD103	10
PercP CD3	10
APC CD45	2,5

- Use the vortex to homogenize the sample.
- Incubate at room temperature and without light for 30 minutes.
- Add 2 mL of CELLWASH and centrifuge to 1800 rpm for 5 minutes at room temperature.
- Decant supernatant and resuspend with 500 µL of CELLWASH.

4.4.4. Machine preparation

- Select CELLQUEST to start the program to do the cytometry.
- Open the file with the name “Plantilla IEL YACINE 2”, to do it go to “FILE” select “Open document” and search for the template.
- Select “Show inspector” clicking “Windows” in the top menu.
- Connect the program to the cytometer selecting “Acquire” and then “Connect to Cytometer”.
- Select “cambio de directorio”, write the name of the sample and press “Choose”.
- Select “Cytometer” from the top menu:
    - Instruments Settings.
    - Open document.
    - Select “Instruments setting Yacine 4-10-2017”.
    - Have the option “Setup” unchecked.

- Start the cytometer pressing “RUN” and “MED”
- Position the tube and select “acquire”. The readings of the sample will begin. Once finished, save the file and print the results.
- Withdraw the tubes of the cytometer.

5. RESULTS AND DISCUSSION

5.1. Percentage of IEL as validation method

The flowing software was used to carry out this part of the study, to do a re-analysis of the results obtained with the flow cytometry.

The first step was to determine the number of IEL out of the total number of cells. This has been performed by graphical inspection of the flow cytometry results. The SSC marker is represented in the ordinate axis and CD45 marker in the abscissa axis. It is well known that the IEL are located at the bottom right position of the graphic because the SSC marker talks about the cell complexity and the CD45 marker is a phosphotyrosine protein found in all the leukocytes.

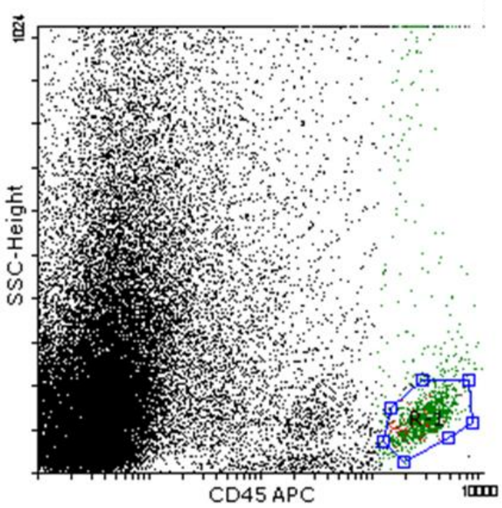


Figure 3: Population selected as IEL.

Once this step is made, these selected cells have to be corroborated as IEL, facing the CD45 and CD103 markers, because the CD103 marker allows us to recognise intraepithelial lymphocytes T.

The reason for not making the selection directly facing CD45 and CD103 markers is the possibility to select dendritic cells as IEL. This possible confusion is due to the CD45 marker being a common antigen to all the leukocytes and the CD103 marker being a common antigen to all the epithelial lymphocytes T, activated cells from the area and a specific group of dendritic cells specialized in the generation of regulatory T lymphocytes.[13, 14]

When making the first selection we found out that the dendritic cells were erased because of being located higher up on the ordinate axis (Figure 3), which shows us that these cells are more complex than lymphocytes and also located more to the left on the axis of abscissa.

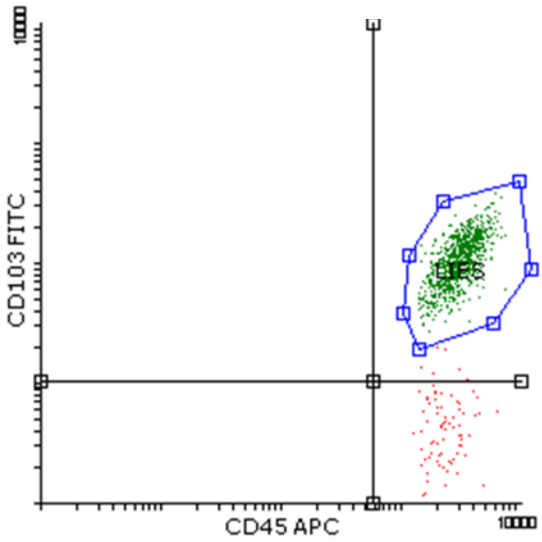


Figure 4: Cells selected that really are IEL.

In the study of IEL as a method of flow cytometry validation, the results proved a correct technique in 95,06% of patients because the percentage of IEL selected on these graphs was higher than the established cut-off. This cut-off value has been selected as 80% based on previous experiences in the “Hospital Universitari Mútua Terrassa”.

5.2. Percentage of IEL as diagnostic marker of celiac disease

Once the results that match the validation approach are chosen, the percentage of IEL within the total number of cells was studied to determine whether it was significant to use it as a biological marker to diagnose celiac disease.

To complete this part of the study, the same process to calculate the IEL like a validation method was used. And once the analysis of all the samples ended, the results obtained were the ones shown on Table 2.

Table 2: A comparative of the percentage of IEL between the group with the results of the duodenum biopsy compatible with a healthy pattern and the results of the ones compatible with celiac disease pattern. The chosen alpha value is 0,05.

	Healthy patients	**Celiac patients**
Average	1,2654	2,9717
Variance	1,5317	8,3273
Grouped variance	6,2637	
Average hypothetical difference	0	
Degrees of freedom	191	
Statistic T	-4,3635	
P(T<=t) one cue	1,0464 E-05	
Critical value of T (one cue)	1,6529	

From a statistical analysis, a T student test has been employed. The initial assumption is that there are not differences between the average value of the two groups, therefore a zero average hypothetical difference has been chosen. The p value is lower than the alpha value, there for exists significant differences between the two groups of patients. However, the average of the celiac disease pattern group of patients was significantly lower than the average found in similar studies.[15, 16]

Finally, a part of the cell population was discovered to be dead cells or cell fragments counted as healthy ones. Therefore, the analysis was repeated deleting this dead or fragmented population.To remove these cellular remnants, the markers SSC and FSC were faced because they are cellular complexity and size markers, respectively. The complexity grows from bottom to top and the size grows from left to right as shown in the graphics (Figure 5). Therefore, the population left on the right of the reference was selected and the area on the left was deleted from the cell count.

The reference was determined based on the down limit of the IEL population because it is known that lymphocytes are small cells (Figure 5). All the points at the left of the cut-off are considered remnants and they are not counted in the total number of cells. This process was performed after applying the validation protocol.

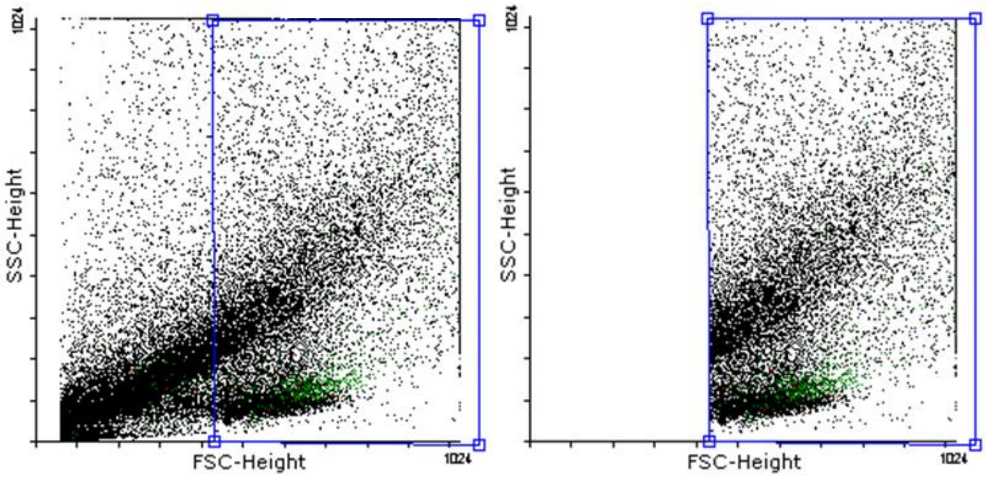


Figure 5: Removal of cell remnants.

Removing this area, higher averages were achieved of both groups because of the decreasing of the total number of cells. The final results are shown on Table 3.

Table 3: Comparison of the percentage of IEL between the group with the results of the duodenum biopsy compatible with a healthy pattern and the results of the ones compatible with celiac disease pattern once the cellular remnants are removed.

	Healthy patients	**Celiac patients**
Average	2,7764	6,665
Variance	7,3012	30,1596
Grouped variance	23,2183	
Average hypothetical difference	0	
Degrees of freedom	191	
Statistic T	5,1650	
P(T<=t) one cue	3,0081E-07	
Critical value of T (one cue)	1,6529	

The results show that significant differences exist between the two groups, healthy patients and celiac patients, without removing the remnant cells (Table 2) and removing them (Table 3). The IEL average in celiac patients is larger in the protocol removing remnants compared to the study where all the cells were counted. Despite increasing the average IEL value in the celiac patients in the protocol removing the remnants, this average value is still lower than the one found in previous studies.[15, 16] This may be caused because the biopsy was repeated in celiac patients after following a gluten-free diet. This diet might help to recover the damage of the duodenum and this could distort the results.

On the other hand, a ROC curve was created to study the sensibility and specificity of the technique and the results obtained. ROC curves are a representation of the sensibility values (ordinate axis) and 1 - specificity (abscissa axis), where all the possible cutting points between the two groups are used, representing this way all the sensibility and specificity values. The value of the area under the curve (AUC), may vary between 0,5 (when the results are totally random) and 1 (when both groups are totally differentiated). It is usually considered an acceptable value when the AUC is bigger than 0,7.[17, 18] In the current study, the ROC curve has an AUC of 0,719 (Figure 6), therefore, it is considered an acceptable result. Further information may be found in Annex 1.

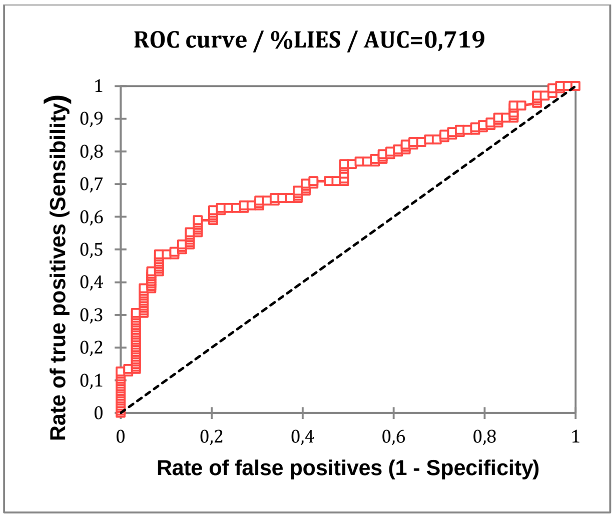


Figure 6: Roc curve to calculate the discriminating capacity of the percentage of IEL in the celiac disease diagnosis.

The Youden Index is a value that maximizes sensibility and specificity for the parameter to be studied. The equation is as follows:

$$\text{YOUDEN INDEX} = \text{SENSIBILITY} + \text{SPECIFICITY} - 1 \quad \text{(Eq. 1)}$$

The closer this value is to 1, the better spacing between groups gives us this parametre.[3, 18] The cut-off for the IEL percentage is established at 3,940 and generates a Youden index of 0,421; herein a 59% of sensibility and a 83,1% of specificity is obtained.

The percentage of IEL as a diagnostic marker is more specific than sensible. This means that following the established criteria, the patients considered with celiac disease most probably will be celiac than the patients considered healthy will be healthy. This is an advantage of the current protocol because the cost for a healthy patient to start a restricted diet is meaningless compared to continuing a usual diet for a celiac patient.

5.3. Transglutaminase ratio and flow cytometry results

In previous studies, it has been observed that there exists a direct correlation between the CD3 and gamma delta markers with celiac disease.[10] CD3- lymphocytes are decreased in celiac disease, while positive gamma delta lymphocytes are larger in celiac patients.

To obtain the results of the number of negative CD3 IEL, the markers CD3 and CD103 were plotted together. This comparison allows us to detect which intraepithelial lymphocytes are CD3 positive and which ones are negative (Figure 7 top).

To determinate the results of the number of positive gamma delta IEL, were compared this two markers: CD3 and TCR gamma delta. (Figure 7 bottom)

Figure 7 shows the results of a celiac pattern and a healthy pattern.

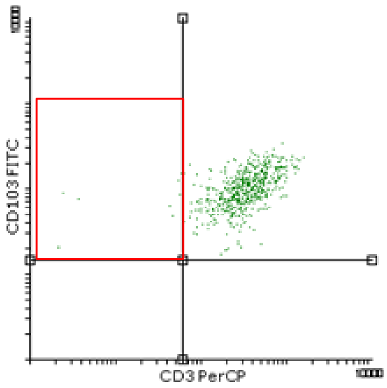
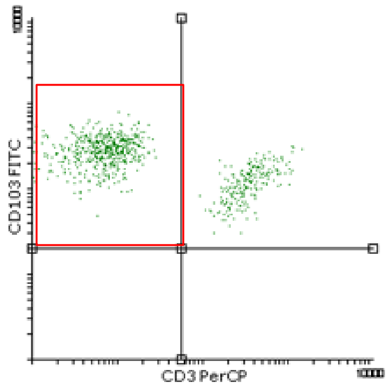
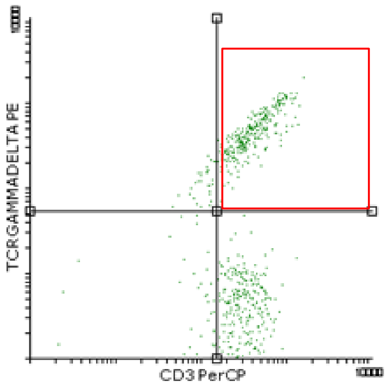
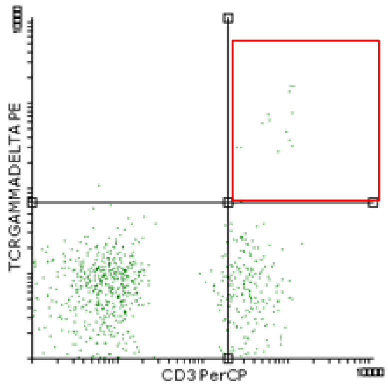
	Celíac pattern	Healthy pattern
CD3-		
Gamma delta+		

Figure 7: Flow cytometry results comparison between patient with celiac pattern and patient with healthy pattern

The cut-off between the groups are:

- For CD3- higher than 10 the patient is considered healthy, under this value, the patient is considered celiac.
- For gamma delta+ smaller than 8,5 the patient is considered healthy, over this value, the patient is considered celiac.

To diagnose a patient like a celiac, it is necessary to present the two values altered, value of negative CD3 decreased and positive gamma delta increased, if it has only one of the parameters altered it will be necessary to evaluate other parameters.

With all the results obtained following this methodology, a comparison was made to search any relation between flow cytometry results and anti-transglutaminase antibodies value.

To realize this comparison, the serological values of the patients were selected. If patients had more than one anti-transglutaminase antibody value, the result closest to the day of the biopsy was selected.

The Pearson and Spearman coefficients were used. Pearson coefficient is used to indicate the linearity between two parameters. Spearman coefficient is used to indicate the monotony between two parameters. These two coefficients can have values from -1 to 1. The difference between the coefficients is that in the Pearson coefficient the difference between the two parameters must remain constant while in the Spearman coefficient it indicates whether the function increases or decreases.[19, 20, 21]

The values near -1 and 1 indicate a maximum correlation, when the value is 1 there exists a direct correlation while when the value is -1 exists an inverse correlation. When the coefficient value is 0, there is not any correlation between the groups. For any other value, there exists a correlation between the groups and the closer the value is to the extremes, the stronger the correlation.[19, 20, 21]

The Pearson value in the comparative between negative CD3 IEL and anti-transglutaminase antibodies was -0,489 and a p value lower than 0,0001. This result indicates that there exists a weak inverse correlation. The Spearman value was -0,732 and indicates a decreasing monotony.

The Pearson value in the comparative between positive gamma delta IEL and anti-transglutaminase antibodies was 0,427 and the Spearman rho (ρ) value was 0,642 and indicates an increasing monotony.

In the two cases the Spearman value is more near to the extremes than the Pearson value, these results indicate that the relationship between the parameters exists but it is not a linear correlation.

Then, the ROC curve was calculated to know how reliable the anti-transglutaminase antibodies value is to determine celiac disease. Further information may be found in Annex 2.

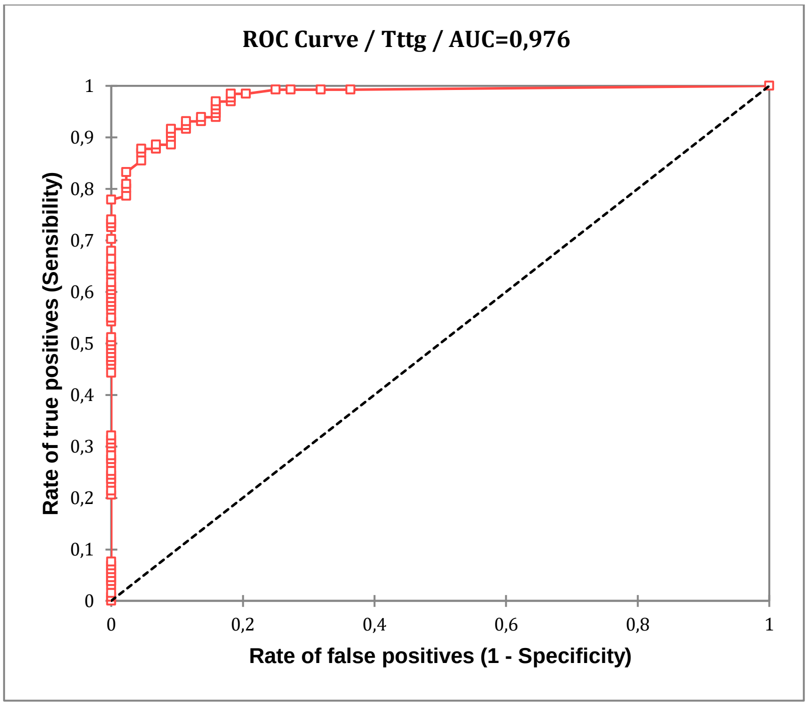


Figure 8: ROC curve to calculate the capacity of anti-transglutaminase antibodies to diagnose celiac disease.

Figure 8 shows that the area under the curve is 0,976 and the maximum possible is 1, this result indicates that anti transglutaminase antibodies is a very good parameter to discriminate between healthy patients and celiac patients.

According to the currently analysed data, the cut-off for anti-transglutaminase antibodies should be 9,2. This value shows the greatest Youden index with a 0,833 value, formed by a sensibility of 87,8% and a specificity of 95,5%.

As discussed in the introduction, there are special cases that with the anti-transglutaminase antibodies value that would be enough to diagnose the patient as a celiac and would allow to discard the realization of the duodenal biopsy. This possibility has been verified in this study because when the value of the anti-transglutaminase antibodies was higher than 17 there was no false positive.

5.4. Percentage of IEL to diagnose results with incomplete pattern

Among the 230 samples, 37 have an incomplete pattern as a result, either due to a decrease in negative CD3 without increase in gamma delta lymphocytes or an increase in gamma delta lymphocytes without a decrease in negative CD3.

Table 4: Flow cytometry results of patients with incomplete patterns.

%IEL	Positive GAMMA DELTA	Negative CD3	Interpretation	Diagnosis	%Lympho
92,91	3,25	8,89	decrease CD3	no celiac	6,34
89,12	5,4	1,53	decrease CD3	celiac	6,5
99,09	7,03	2,42	decrease CD3	no celiac	7,59
92,59	11,79	44,72	Increase GD	no celiac	0,01
87,68	11,2	11,3	Increase GD	no celiac	0,13
93,1	28,85	28,96	Increase GD	no celiac	0,27
89,96	10,7	21,1	Increase GD	no celiac	0,3
91,49	31,94	13,61	Increase GD	celiac	0,45
97,5	34,4	9,81	Increase GD	no celiac	0,58
86,68	15,3	11,7	Increase GD	no celiac	0,69
96,33	16,91	9,19	Increase GD	celiac	0,71
96,84	14	33,22	Increase GD	no celiac	0,77
87,33	26,57	26,94	Increase GD	celiac	0,78
87,79	13,3	47,5	Increase GD	no celiac	1,12
95,64	15,7	28,1	Increase GD	no celiac	1,28
82,88	19,88	46,5	Increase GD	no celiac	1,53
95,61	14,55	11,85	Increase GD	celiac	1,55
91,11	48,07	10,76	Increase GD	celiac	1,59
95,16	21,4	30,6	Increase GD	no celiac	1,61
89,91	13,5	10,1	Increase GD	no celiac	1,71
94,2	37,2	12,3	Increase GD	celiac	1,95
93,85	21,2	29,7	Increase GD	no celiac	1,99
95,09	37,91	21,09	Increase GD	no celiac	2,07
93,09	15,5	12,9	Increase GD	celiac	2,2
89,94	11,16	40,7	Increase GD	no celiac	2,47
91,19	20,1	22,72	Increase GD	no celiac	2,54

95,45	26,61	25,03	Increase GD	celiac	2,62
94,86	16,5	38,16	Increase GD	no celiac	2,74
93,15	47,7	19,6	Increase GD	celiac	3,03
90,5	12,76	13,18	Increase GD	no celiac	3,6
94,22	17,44	32,26	Increase GD	no celiac	3,71
97,32	28,78	21,94	Increase GD	no celiac	3,87
97,37	14	10,8	Increase GD	no celiac	4,4
85,84	43	17,3	Increase GD	celiac	4,77
93,44	16	20,8	Increase GD	celiac	5,05
93,65	31,3	21,8	Increase GD	celiac	5,53
91,53	12,85	43,9	Increase GD	celiac	5,85

The diagnosis has been performed following the criteria explained in the introduction with the diagnostic criteria explained in the introduction.

Of the 37 patients, following the diagnostic criteria with respect to the percentage of intraepithelial lymphocytes obtained (3,94), 25 would have been diagnosed correctly. This result, 25 of 37 represent a 70,27%, value very similar to the obtained with the group with complete pattern. With these results, the value of the percentage of intraepithelial lymphocytes, can be used as an additional parameter to diagnose patients, as a celiac, with incomplete pattern biopsies results.

6. CONCLUSIONS

According to the results that has been explained in this thesis, it can be concluded that:

- The inclusion of IEL validation produces more accurate results than those obtained without this procedure.
- The IEL percentage is another biomarker to consider when diagnosing a patient with celiac disease.
- The anti-transglutaminase antibodies value has a direct relation with the positive gamma delta IEL value and an inverse relation with the negative CD3 IEL value.
- The IEL percentage is a biomarker to consider when a patient has an incomplete pattern as a result of the duodenal biopsy.

With these conclusions, it could be stated that the main goal has been achieved: Study the percentage of intraepithelial lymphocytes (IEL) in possible celiac patients. The secondary goals have also been achieved.

Finally, it should be noted the importance of continuing to study this area to obtain a higher sample population and continue validating the results. These results only are applicable in the sample population but it is still not comparably to the general population.

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8. ANNEXES

8.1. Annex 1: Results calculation curve ROC % IEL

Table 5: Results used to create the ROC curve of the LIES percentage as a biomarker for the celiac diagnosis.

92,25	4,8	41,8	NORMAL
93,92	4,32	43,24	NORMAL
87,98	7,41	37,87	NORMAL
94,7	1,52	25,4	NORMAL
93,68	10	52,8	NORMAL
98,74	4,7	9,17	NORMAL
97,42	10	36	NORMAL
84,01	2,3	38,03	NORMAL
87,49	6,83	16,85	NORMAL
96,8	3,44	46,98	NORMAL
88,17	6,05	39,09	NORMAL
89,11	6,88	38	NORMAL
86,79	3,95	19,16	NORMAL
92,31	4	50,4	NORMAL
84,1	8	18	NORMAL
92,73	6,53	60,28	NORMAL
89,58	1,7	69,35	NORMAL
80,31	6,77	38,25	NORMAL
94,67	7,89	42,32	NORMAL
95,34	6,39	33,8	NORMAL
97,65	8,6	18,7	NORMAL
89,62	0,28	20,17	NORMAL
96,36	4,2	9,83	NORMAL
97,99	4,92	32,68	NORMAL
97,15	0,98	20,82	NORMAL
94,11	7,35	56	NORMAL
91,34	7,38	16,44	NORMAL
96,23	6,8	34,44	NORMAL
91,29	7,7	38,95	NORMAL
90,67	5,68	38,75	NORMAL
94,99	0,21	26,5	NORMAL
94,98	6,3	27,9	NORMAL
80,36	3,6	35,53	NORMAL
90,61	6,09	16,55	NORMAL
95,73	8,1	30	NORMAL
96,95	0,1	33,5	NORMAL
93,9	12,58	55,33	NORMAL
98,37	2,3	22,9	NORMAL
92,49	6,8	18,38	NORMAL
94,37	1,1	20,1	NORMAL
94,24	2,74	46,52	NORMAL

89,85	2,81	57,08	NORMAL
95,83	3,4	25,8	NORMAL
94,79	8,03	30,03	NORMAL
97,25	5,66	24,2	NORMAL
92,71	9,4	41	NORMAL
98,52	7,56	27,48	NORMAL
96,86	2,99	8,38	NORMAL
86,49	9,29	11,38	NORMAL
92,52	2,64	19,69	NORMAL
96,6	3,88	10,59	NORMAL
98,09	6,63	11,39	NORMAL
94,81	3,01	28,8	NORMAL
95,16	8,8	27,7	NORMAL
96,3	8,26	40,87	NORMAL
97,53	3,39	43,02	NORMAL
95,45	0,95	24,4	NORMAL
99,03	7,57	15,78	NORMAL
91,41	8,1	49,78	NORMAL
97,27	26,44	1,76	CELIAC PATTERN
95,36	58	6,1	CELIAC PATTERN
80,46	29,16	6,63	CELIAC PATTERN
97,21	25	1,3	CELIAC PATTERN
98,41	23,2	3,29	CELIAC PATTERN
91,62	26	1	CELIAC PATTERN
90,82	15,67	2,81	CELIAC PATTERN
87,49	36,06	0,72	CELIAC PATTERN
97,02	32,17	2,85	CELIAC PATTERN
92,93	28,7	5,5	CELIAC PATTERN
81,37	26,3	1,22	CELIAC PATTERN
97,52	37	0,5	CELIAC PATTERN
95,63	63,1	4,5	CELIAC PATTERN
96,27	34,3	1,59	CELIAC PATTERN
89,72	46	2,82	CELIAC PATTERN
89,78	19,38	2,2	CELIAC PATTERN
80,22	28	3,9	CELIAC PATTERN
93,39	33	1,6	CELIAC PATTERN
85,26	17,1	1,3	CELIAC PATTERN
96,47	42,6	1,45	CELIAC PATTERN
90,5	24,35	0,57	CELIAC PATTERN
95,5	39,26	6,43	CELIAC PATTERN
90,43	36,97	2,05	CELIAC PATTERN
95,7	46,9	3,6	CELIAC PATTERN
88,89	46,31	0,94	CELIAC PATTERN
82,25	31,9	5,07	CELIAC PATTERN
80,82	40,3	1,63	CELIAC PATTERN
86,91	33	1,7	CELIAC PATTERN
96,93	60,26	1,28	CELIAC PATTERN

95,41	46,8	1,94	CELIAC PATTERN
89,29	22,8	1,68	CELIAC PATTERN
94,3	27,12	5,03	CELIAC PATTERN
94,94	42,39	1,96	CELIAC PATTERN
85,9	21	1	CELIAC PATTERN
92,04	21,4	5,4	CELIAC PATTERN
85,26	29,28	2,48	CELIAC PATTERN
93,45	33,7	2,61	CELIAC PATTERN
96,97	15,46	0,31	CELIAC PATTERN
93,96	27	0,85	CELIAC PATTERN
92,97	19,34	8,87	CELIAC PATTERN
98,48	76,4	2,8	CELIAC PATTERN
94,86	31,79	7,05	CELIAC PATTERN
97,96	73,6	2,14	CELIAC PATTERN
91,91	48,54	4,65	CELIAC PATTERN
90,32	22,3	2,69	CELIAC PATTERN
91,67	27,27	1,11	CELIAC PATTERN
86,08	38,2	4,91	CELIAC PATTERN
97,67	41	1,4	CELIAC PATTERN
93,43	46,19	1,07	CELIAC PATTERN
91,31	33,8	2,61	CELIAC PATTERN
92,06	30	0,8	CELIAC PATTERN
87,85	39	2	CELIAC PATTERN
93,3	43,1	3,74	CELIAC PATTERN
93,51	51,6	1,07	CELIAC PATTERN
97,73	20,39	2,14	CELIAC PATTERN
94,87	35,8	0,32	CELIAC PATTERN
81,75	31,02	4,38	CELIAC PATTERN
96,61	31,3	1,5	CELIAC PATTERN
92,02	34	2,1	CELIAC PATTERN
86,81	53,39	1,15	CELIAC PATTERN
92,72	44,2	4,8	CELIAC PATTERN
90,92	16,4	0,81	CELIAC PATTERN
84,57	18,3	1,8	CELIAC PATTERN
96,26	9,31	5,07	CELIAC PATTERN
84,22	34,62	2,3	CELIAC PATTERN
97,57	11,4	1	CELIAC PATTERN
81,02	36,49	1,37	CELIAC PATTERN
88,96	23	6,1	CELIAC PATTERN
91,71	20,24	1,2	CELIAC PATTERN
93,95	19,4	5,25	CELIAC PATTERN
91,41	38	4,3	CELIAC PATTERN
81,2	24,5	1,5	CELIAC PATTERN
87,45	32,12	0,91	CELIAC PATTERN
87,76	30,3	1	CELIAC PATTERN
97,74	55,13	3,03	CELIAC PATTERN
95,25	37,54	2,73	CELIAC PATTERN

94,84	25,6	3,13	CELIAC PATTERN
97,34	46,43	1,62	CELIAC PATTERN
99,51	26,14	9,28	CELIAC PATTERN
92,55	26,03	0,98	CELIAC PATTERN
95,8	13,7	0,52	CELIAC PATTERN
82,2	19,27	2,32	CELIAC PATTERN
91,12	48,8	2,4	CELIAC PATTERN
93,3	22,47	2,58	CELIAC PATTERN
83,11	22,02	1,32	CELIAC PATTERN
93,6	48	1,63	CELIAC PATTERN
93,82	14,91	3,43	CELIAC PATTERN
98,13	56,2	3,8	CELIAC PATTERN
89,68	48,98	0,2	CELIAC PATTERN
87,11	38	0,69	CELIAC PATTERN
95,55	12,9	0,73	CELIAC PATTERN
88,99	30,5	2,9	CELIAC PATTERN
95,73	33	1	CELIAC PATTERN
89,06	23,9	0,75	CELIAC PATTERN
96,95	35,7	7,46	CELIAC PATTERN
93,49	16,35	2,35	CELIAC PATTERN
85,77	17,8	1,4	CELIAC PATTERN
89,45	40,29	3,25	CELIAC PATTERN
96,22	10,79	6,33	CELIAC PATTERN
90,38	47,01	1,44	CELIAC PATTERN
85,53	9,14	2,25	CELIAC PATTERN
85,86	33,2	1,78	CELIAC PATTERN
93,13	31,61	2,69	CELIAC PATTERN
85,35	16,5	1,7	CELIAC PATTERN
86,22	48,22	2,13	CELIAC PATTERN
81,43	49,21	5,65	CELIAC PATTERN
88,45	47	0,56	CELIAC PATTERN
94,19	34,7	2,25	CELIAC PATTERN
95,08	35,76	1,37	CELIAC PATTERN
85,12	44,7	1,76	CELIAC PATTERN
90,31	40,1	1,89	CELIAC PATTERN
95,12	17,9	2,8	CELIAC PATTERN
97,72	34	6,06	CELIAC PATTERN
91,5	43	1,7	CELIAC PATTERN
80,58	19,8	0,5	CELIAC PATTERN
95,92	29	3	CELIAC PATTERN
90,2	31,69	0,77	CELIAC PATTERN
95,95	24,5	1,57	CELIAC PATTERN
89,47	21,3	1,7	CELIAC PATTERN
88,92	31,2	0,88	CELIAC PATTERN
84,91	18,1	0,88	CELIAC PATTERN
94,7	36,5	0,42	CELIAC PATTERN
90,77	33,48	1,09	CELIAC PATTERN

80,71	38,14	0,64	CELIAC PATTERN
98,76	36,9	1,18	CELIAC PATTERN
93,94	16,8	0,42	CELIAC PATTERN
93,14	33,8	0,53	CELIAC PATTERN
93,5	43,37	0,95	CELIAC PATTERN
92,43	48,6	1,23	CELIAC PATTERN
92,79	28	0,75	CELIAC PATTERN
91,63	13,2	1,7	CELIAC PATTERN
91,77	36,27	0,76	CELIAC PATTERN
96,35	80,8	0,52	CELIAC PATTERN
92,88	17,58	0,5	CELIAC PATTERN

8.2. Annex 2: Results for the calculation of the ROC curve, transglutaminase antibodies

Table 6: Results used to create the ROC curve of the anti-transglutaminase antibodies like a biomarker for the celiac disease diagnosis.

Gamma delta	CD3-	Tttg	Interpretation
0.1	33.5	0.1	N
0.21	26.5	0.1	N
0.95	24.4	1.3	N
0.98	20.82	0.1	N
1.1	20.1	0.1	N
1.52	25.4	0.1	N
1.7	69.35	0.1	N
2.3	22.9	0.1	N
2.3	38.03	0.1	N
2.64	19.69	0.1	N
2,74	46,52	0,1	N
2,99	8,38	0,1	
3,01	28,8	0,1	N
3,25	8,89	0,1	
3.39	43.02	0.5	N
3.4	25.8	0.1	N
3.44	46.98	0.4	N
3.6	35.53	0.1	N
3.88	10.59	0.1	N
3.95	19.16	3.5	N
4	50,4	0,1	N
4,2	9,83	0,3	
4,32	43,24	16	N
4,7	9,17	2	
4.8	41.8	0.1	N
4,92	32,68	0,1	N
5,4	1,53	0,1	
5.66	24.2	0.1	N
5.68	38.75	0.1	N
6.05	39.09	0.8	N
6.09	16.55	0.1	N
6.3	27.9	0.1	N
6.53	60.28	8.3	N
6.63	11.39	0.3	N
6.77	38.25	0.1	N
6.8	18.38	0.5	N
6.8	34.44	5.1	N
6.83	16.85	0.1	N
6,88	38	11	N
7,03	2,42	1,2	
7.35	56	0.3	N
7,56	27,48	0,1	N

7.57	15.78	0.1	N
7.7	38.95	0.1	N
7.89	42.32	4	N
8	18	0.2	N
8.03	30.03	0.1	N
8.1	30	9	N
8.1	49.78	0.2	N
8,26	40,87	0,1	N
8,6	18,7	0,1	
9,14	2,25	128	P
9,29	11,38	0,1	
9,31	5,07	1,1	P
9,4	41	0,8	
10	36	0,8	
10	52,8	0,3	
10,7	21,1	8	
10,79	6,33	58	P
11,16	40,7	0,7	
11,2	11,3	9,8	
11,79	44,72	0,5	
12,58	55,33	0,1	
12,76	13,18	0,1	
12,85	43,9	33	
13,2	1,7	23	P
13,3	47,5	0,1	
13,5	10,1	0,9	
14	10,8	0,1	
14	33,22	0,6	
14,55	11,85	30	
14,91	3,43	9,2	P
15,3	11,7	0,4	
15.46	0.31	75	P
15,67	2,81	17	P
15,7	28,1	0,1	
16	20,8	57	
16.35	2.35	80	P
16.4	0.81	128	P
16,5	1,7	1,2	P

16,5	38,16	0,3	
16.8	0.42	128	P
16.91	9.19	2	P
17,1	1,3	17	P
17,44	32,26	0,1	
17.58	0.5	80	P
17.8	1.4	23	P
17.9	2.8	3.6	P
18.1	0.88	250	P
18.3	1.8	105	P
19.27	2.32	80	P
19.34	8.87	2.7	P
19.38	2.2	24	P
19.4	5.25	0.5	P
19,8	0,5	42	P
20,1	22,72	0,4	
20.24	1.2	128	P
20.39	2.14	7.4	P
21	1	19	P
21,2	29,7	0,1	
21.3	1.7	110	P
21,4	5,4	58	P
21,4	30,6	2,3	
22.02	1.32	20	P
22.3	2.69	80	P
22.47	2.58	128	P
22.8	1.68	80	P
23	6.1	7.4	P
23.2	3.29	11	P
23.9	0.75	115	P
24.35	0.57	107	P
24.5	1.5	80	P
24.5	1.57	159	P
25.6	3.13	43	P
26	1	128	P
26.03	0.98	66	P
26.14	9.28	5.3	P
26.3	1.22	51	P
26,44	1,76	70	P
26,57	26,94	6,8	
26,61	25,03	85	
27	0.85	128	P
27.12	5.03	15	P
27.27	1.11	27	P
28	0.75	251	P
28	3.9	80	P
28,7	5,5	75	P
28,78	21,94	5,3	

28,85	28,96	0,1	
29	3	18	P
29.16	6.63	0.1	P
29.28	2.48	98	P
30	0.8	157	P
30.3	1	168	P
30.5	2.9	60	P
31.02	4.38	128	P
31.2	0.88	14	P
31,3	1,5	20	P
31,3	21,8	22	
31.69	0.77	128	P
31.79	7.05	2.5	P
31,9	5,07	11	P
31,94	13,61	5,5	
32.12	0.91	24	P
32.17	2.85	80	P
33	1	62	P
33	1.6	2.9	P
33	1.7	80	P
33.2	1.78	29	P
33.48	1.09	128	P
33.7	2.61	60	P
33.8	0.53	17	P
33.8	2.61	8.6	P
34	2.1	138	P
34	6.06	28	P
34.3	1.59	63	P
34.4	9.81	13	P
34.62	2.3	60	P
34.7	2.25	128	P
35.7	7.46	120	P
35.76	1.37	80	P
35.8	0.32	128	P
36.06	0.72	128	P
36.27	0.76	4.9	P
36.49	1.37	59	P
36.5	0.42	110	P
36.9	1.18	28	P
36.97	2.05	128	P
37	0,5	22	P
37,2	12,3	9,3	
37,54	2,73	65	P
37,91	21,09	0,1	
38	0.69	123	P
38	4.3	120	P
38.14	0.64	60	P
39	2	10	P
39	2	10	P
39.26	6.43	6.8	P
40,1	1,89	80	P

40.29	3.25	22	P
40.3	1.63	55	P
41	1.4	64	P
42.39	1.96	128	P
42.6	1.45	85	P
43	1,7	15	P
43	17,3	69	
43.1	3.74	4.4	P
43.37	0.95	273	P
44.2	4.8	178	P
44.7	1.76	128	P
46	2.82	250	P
46.19	1.07	50	P
46.31	0.94	220	P
46.43	1.62	119	P
46.8	1.94	13	P
46.9	3.6	17	P
47	0.56	100	P
47,01	1,44	100	P
47,7	19,6	13	
48	1,63	80	P
48,07	10,76	0,8	
48.22	2.13	80	P
48.54	4.65	80	P
48.6	1.23	95	P
48.8	2.4	80	P
48.98	0.2	16	P
49.21	5.65	11	P
51.6	1.07	13	P
53.39	1.15	80	P
55.13	3.03	31	P
56.2	3.8	69	P
58	6.1	17	P
60.26	1.28	25	P
63.1	4.5	112	P
73.6	2.14	20	P
76.4	2.8	22	P
80,8	0,52	128	P